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***I nitriti nel cuore di  
mammiferi e non-mammiferi:  
risorsa fisiologica di ossido nitrico e  
importante molecola segnale***

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The ubiquitous anion nitrite ( $\text{NO}_2^-$ ) has recently emerged as an endocrine storage form of nitric oxide (NO) and a signalling molecule that mediates a number of biological responses. Although the role of NO in regulating cardiac function has been investigated in depth, the physiological signalling effects of nitrite on cardiac function have only recently been explored.

We evaluated nitrite influence on cardiac performance in the perfused beating heart of four different vertebrates: the temperate red-blooded *Anguilla anguilla*, the Antarctic stenotherm, hemoglobinless *Chionodraco hamatus* (icefish), the frog *Rana esculenta* and the Wistar rat. We showed that, under basal conditions, in all animals nitrite influences cardiac mechanical performance, inducing negative inotropism in eel, frog and rat, while being a positive inotrope in *C. hamatus*. In all species, these responses parallel the inotropic effects of authentic NO. We also demonstrated that the nitrite-dependent inotropic effects are i) dependent from NO synthase (NOS) activity in fish; ii) sensitive to NO scavenging in frog and rat; iii) cGMP/PKG-dependent in eel, frog and rat. Results suggest that nitrite is an integral physiological source of NO and acts as a signalling molecule in vertebrate hearts, exerting relevant inotropic actions through different species-specific mechanisms.

These comparative studies may pave the way towards an integrated systemic approach concerned with the role of nitrite/NO signalling in

vertebrate cardiac physiology, which is also essential for medically-oriented studies and clinical applications.

## ***PREMESSA E SCOPO DELLA TESI***

Negli ultimi anni è stato dimostrato un nuovo meccanismo di produzione dell'ossido nitrico (NO), Ossido Nitrico Sintasi (NOS)-indipendente, che consiste nella riconversione dei nitriti ( $\text{NO}_2^-$ ) a NO (Cosby et al., 2003). Tale sintesi alternativa può derivare da tre diversi meccanismi: riduzione enzimatica, disproporzionazione o riduzione acida dei nitriti (Ferrari et al., 1996; Cosby et al., 2003). L'attività nitrito-reduttasica è stata rilevata in specifici enzimi, come la glutatione-S-transferasi (Hill et al., 1992), la xantina ossidoreduttasi (Millar et al., 1998), il citocromo P-450 (Kozlov et al., 2003), la deossiemoglobina (Gladwin et al., 2004), la eNOS in assenza di ossigeno (Gautier et al., 2006). Le condizioni perché tali reazioni avvengano si hanno tipicamente a pH acido e in carenza di ossigeno e si verificano dunque in stati patologici, quali l'ischemia (Duranski et al., 2005; Webb et al., 2004).

Tuttavia, da recenti studi è emerso che l'anione nitrito gioca un ruolo chiave non solo in condizioni patologiche ma anche in condizioni fisiologiche: esso rappresenta, infatti, una fondamentale molecola segnale in grado di modulare la stimolazione della guanilato ciclastasi solubile (sGC), l'attività del citocromo P-450 e l'espressione di due importanti proteine, heat shock protein 70 (Hsp 70) e heme oxygenase-1 (HO-1) (Bryan et al., 2005).

Alla luce di tali evidenze sperimentali, scopo del presente lavoro di tesi è stato quello di analizzare gli effetti cardiaci dei nitriti in condizioni basali, nonché i meccanismi biologici coinvolti, su quattro specie diverse, *Anguilla anguilla*, *Chionodraco hamatus* (icefish), *Rana esculenta* e ratto

Wistar, il cui cuore è soggetto ad un importante controllo autocrino-paracrino mediato dallo NO. A tal fine sono stati utilizzati cuori di anguilla, icefish e rana, isolati e perfusi secondo la metodica “working” messa a punto nei nostri laboratori, e cuori di ratto, isolati e perfusi secondo la metodica *Langendorff*. Questi preparati consentono di lavorare in condizioni di perfusione simili a quelle *in vivo* e risultano idonei ad effettuare studi fisiofarmacologici scevri da artefatti sperimentali.

La scelta di tali modelli ha permesso di indagare gli effetti dei nitriti su animali con un design cardiaco ben diverso. Questi modelli presentano importanti differenze sia nell’architettura ventricolare (trabecolata in icefish e rana; trabecolata e solo in parte compatta in anguilla; del tutto compatta in ratto), sia nell’assetto vasale (le coronarie sono totalmente assenti in tutti i modelli presi in esame, eccetto nel ratto). Il fatto di utilizzare dei cuori avascolari assume particolare importanza negli studi sullo NO: modelli senza coronarie permettono, infatti, di valutare gli effetti diretti sul miocardio senza interferenze da parte della porzione vasale che, com’è noto, è estremamente reattiva nei confronti dello NO.

Inoltre le specie prese in esame presentano peculiarità per niente trascurabili. Sia l’anguilla che la rana rappresentano, infatti, esemplari campioni di tolleranza nei confronti di prolungata ipossia e acidosi, vivendo in un ambiente soggetto ad importanti variazioni di O<sub>2</sub>. Al contrario l’icefish rappresenta un caso unico di estrema stenotermia, abitando nelle acque antartiche stabilmente ghiacciate e riccamente ossigenate. Per di più,



l'icefish rappresenta un'esclusiva opportunità per investigare la via NO/nitriti poiché è un naturale modello knockout per l'emoglobina, proteina chiave nell'omeostasi dello NO in grado di agire sia da scavenger che da generatore di NO.

Tale analisi comparata potrebbe essere di notevole importanza non solo per comprendere l'attività biologica dei nitriti nel modulare le normali funzioni del cuore di diversi vertebrati, ma anche per dare avvio ad importanti scoperte mediche e ad eventuali applicazioni cliniche.

## ***INTRODUZIONE***

## 1. OSSIDO NITRICO

Lo NO è uno dei più importanti mediatori biochimici conosciuti: la maggior parte delle cellule di un organismo sintetizzano NO, una molecola semplice ma coinvolta nella regolazione dei sistemi cardiovascolare, immunitario e nervoso. Identificato inizialmente come fattore EDRF (Endothelium-Derived Relaxing Factor; Ignarro et al., 1987), lo NO è stato in seguito riconosciuto come un importante modulatore di diverse funzioni, che includono la neurotrasmissione, l'inibizione dell'adesione e aggregazione piastrinica, la proliferazione cellulare, la regolazione dell'attività citotossica del macrofago (Moncada et al., 1991; Radomski & Moncada, 1993; Vane, 1994), nonché la modulazione della funzione cardiaca (Shah et al., 1999; Massion et al., 2003).

Lo NO è una molecola di natura radicalica, lipofilica ed altamente reattiva, la cui emivita è dell'ordine dei secondi: il gas è perciò in grado di agire sulla stessa cellula che lo ha prodotto o vicino al sito di produzione, ha cioè azione autocrina-paracrina (Shah & MacCarthy, 2000). Esso può interagire con altri radicali, ioni metallici di transizione e nucleofili come i tioli e le amine, producendo così effetti diretti o indiretti (Wink & Mitchell, 1998; Grisham et al., 1999).

Gli effetti diretti spesso coinvolgono interazioni tra lo NO e i complessi metallici. Le reazioni tra lo NO e le proteine contenenti il gruppo eme sono fisiologicamente più rilevanti e includono interazioni con la sGC e

il citocromo P450. Inoltre, lo NO può interagire con le proteine non-eme contenenti ferro o contenenti zinco.

Gli effetti indiretti sono mediati da specie nitrogeno reattive prodotte dall'interazione tra lo NO e l'ossigeno o i radicali superossido (Pellat et al., 1990).

Gli effetti indotti dallo NO risultano concentrazione-dipendente: a basse dosi (<1μM) prevalgono gli effetti diretti mentre ad alte dosi (>1μM) sono predominanti gli effetti indiretti (reviewed in Cerra & Pellegrino, 2007).

## **2. BIOSINTESI DELLO NO**

La biosintesi dello NO avviene principalmente attraverso la trasformazione dell'amminoacido L-arginina in L-citrullina operata dalle NOS (Moncada & Higgs, 1993; Bredt et al., 1991). La reazione comporta l'ossidazione del gruppo guanidinico della L-arginina in un processo che utilizza 5 elettroni e 5 cofattori, NADPH, FAD, FMN, tetraidrobiopterina e Ca<sup>2+</sup>-calmodulina.

Sono state identificate tre isoforme dell'enzima NOS: neuronale (nNOS o NOS I), inducibile (iNOS o NOS II) ed endoteliale (eNOS o NOS III), ognuna delle quali è il prodotto di geni distinti (Nathan & Xie, 1994). La eNOS e la nNOS sono costitutivamente espresse e la loro attività è regolata dalle variazioni della concentrazione di Ca<sup>2+</sup> citosolico (Knowles et al., 1989; Palmer & Moncada, 1989); la iNOS, invece, è scarsamente presente a

livello basale e la sua espressione è indotta a livello trascrizionale da stimoli specifici, inoltre la sua attività non è  $Ca^{2+}$ -dipendente (Schulz et al., 1992).

Le caratteristiche delle tre isoforme dell'enzima sono riassunte nella *tabella 1*.

	<b>nNOS</b>	<b>iNOS</b>	<b>eNOS</b>
	NOS neuronale o NOS-I	NOS inducibile o NOS-II	NOS endoteliale o NOS-III
<b>Peso molecolare</b>	~160 kDa	~131 kDa	~133 kDa
<b>Tessuti</b>	Sistema nervoso Muscolo scheletrico Isole pancreatiche Endometrio Macula densa	Macrofagi Cuore Fegato Muscolo liscio Endotelio	Endotelio Cervello Epitelio
<b>Localizzazione subcellulare</b>	Citosolica	Citosolica e particolata	Particolata a livello caveolare
<b>Funzioni</b>	Neurotrasmissione Interazioni tubulo-glomerulari Motilità intestinale	Antimicrobico Citotossico Infiammazione	Rilassamento vascolare Adesione piastrinica Angiogenesi
<b>Inibitori</b>	N-Methyl-L-arginine N-Nitro-L-arginine 7-Nitroindazole	Aminoguanidine S-Benzylisothiourea L-N6-(1-Iminoethyl) lysine	N-Methyl-L-arginine N-Nitro-L-arginine 7-Nitroindazole

**Tabella 1.**

Inoltre, in diversi tipi cellulari è stata evidenziata anche una isoforma mitocondriale (mtNOS) che corrisponde ad una variante dell'isoforma neuronale (nNOS- $\alpha$ ). Essa è, infatti, codificata dagli stessi geni nucleari che codificano per la nNOS ed è poi traslocata a livello mitocondriale (reviewed in Finocchietto et al., 2009).

I membri della famiglia delle NOS sono, tra gli enzimi conosciuti, i più altamente regolati. La regolazione della sintesi di NO avviene a diversi livelli: disponibilità di cofattori e calcio, modificazioni post-trasduzionali ed

interazioni proteina-proteina (Bredt, 2003). Inoltre, la localizzazione tissutale, cellulare e sub-cellulare aggiunge un meccanismo regolatorio fondamentale alla produzione ed agli effetti dello NO; infatti, la natura altamente reattiva e diffusiva dello NO richiede che gli enzimi siano localizzati in prossimità dei target (Barouch et al., 2002).

La diversa localizzazione subcellulare della eNOS e della nNOS rende conto della regolazione autocrina e paracrina esercitata dallo NO sulla funzionalità cardiaca. La eNOS è localizzata a livello delle cellule endoteliali endocardiche e coronariche e nei cardiomiociti (Feron et al., 1996), mentre la nNOS è localizzata a livello delle terminazioni nervose autonome, nei gangli e nei cardiomiociti (Danson et al., 2005; Xu et al., 1999).

Recenti scoperte riportano la presenza sia della eNOS che della nNOS a livello dei distretti subcellulari nei cardiomiociti (Feron et al., 1996; Xu et al., 1999; Williams et al., 2006) dove si suppone siano accoppiate a distinte molecole effettrici e inducano effetti diversi in seguito all'attivazione enzimatica. La eNOS è maggiormente localizzata nelle *caveolae* dove si co-localizza con la caveolina-3, una proteina strutturale specifica delle *caveolae* dei cardiomiociti (reviewed in Shaul, 2002). La nNOS è stata inizialmente localizzata a livello del reticolo sarcoplasmatico (SR) (Xu et al., 1999) sebbene, successivamente, sia stata ritrovata associata alle proteine della membrana sarcolemmale (Williams et al., 2006; Xu et al., 2003).

Lo NO derivante dalla eNOS sembra essere coinvolto nella modulazione della risposta inotropica conseguente allo stretch. Gli effetti autocrini mediati invece dallo NO derivante dalla nNOS comprendono la modulazione dell'inotropismo, del rilassamento e della relazione forza-frequenza. Tali effetti possono cambiare notevolmente in caso di patologie cardiovascolari in cui sia l'espressione sia l'attività delle NOS sono alterate (reviewed in Seddon et al., 2007).

### **3. MECCANISMO D'AZIONE DELLO NO**

Il principale target dello NO generato in condizioni basali è la sGC che viene attivata con conseguente incremento intracellulare di cGMP (Shah & MacCarthy, 2000; Schmidt et al., 1993; Lincoln & Cornwell, 1993). Inoltre, lo NO può esercitare azioni cGMP-indipendenti reagendo direttamente con canali ionici, enzimi, proteine contenenti gruppi eme e specie reattive dell'ossigeno (Davis et al., 2001). Tra le vie cGMP-indipendenti più studiate vengono descritte l'inibizione della citocromo c ossidasi (Torres et al., 1995) e una nuova modificazione post-trasduzionale indotta da NO: la nitrosilazione dei residui cisteinici o S-nitrosilazione (Martinez-Ruiz & Lamas, 2004).

#### **3.1 Attivazione della guanilato ciclasi**

Lo NO si lega al gruppo prostetico eme della sGC che nella forma nitrosilata catalizza la sintesi di cGMP (Arnold et al., 1977). La generazione di cGMP porta all'attivazione di una serie di proteine cGMP-dipendenti, tra

cui chinasi, fosfodiesterasi e alcuni canali di membrana (Lincoln, 1983; Whalin et al., 1988).

La stimolazione delle protein chinasi cGMP-dipendenti (G chinasi) è importante nel controllo dell'omeostasi del  $\text{Ca}^{2+}$ . Esse, infatti, modulano negativamente la  $[\text{Ca}^{2+}]_i$  e permettono quindi un controllo a feedback negativo tra i sistemi dello NO e del  $\text{Ca}^{2+}$ : la generazione di NO, stimolata dall'aumento di  $[\text{Ca}^{2+}]_i$ , limita a sua volta questo aumento. Questo si traduce, a livello cardiaco, in un effetto inotropo negativo per alte concentrazioni di NO e in un effetto inotropo positivo, invece, a basse concentrazioni (Massion et al., 2003).

Inoltre, il cGMP agisce anche in altri modi, non mediati dall'attivazione di G chinasi: esso stimola direttamente l'attività idrolitica di diverse fosfodiesterasi, attive sia nell'idrolizzare il cGMP stesso che il cAMP. Questo meccanismo permette, invece, un controllo a feedback negativo dell'eccessiva espansione del segnale di NO/cGMP.

Infine, il cGMP aumenta la probabilità di apertura di alcuni specifici canali di membrana permeabili a  $\text{Na}^+$  e  $\text{Ca}^{2+}$ .

### **3.2 Inibizione della citocromo c ossidasi**

La citocromo c ossidasi è il complesso terminale della catena di trasporto mitocondriale cui si lega l'ossigeno che viene consumato durante la respirazione.

Lo NO si lega reversibilmente alla citocromo c ossidasi sullo stesso sito in cui si lega l'ossigeno, dunque i due gas competono per lo stesso



enzima. In condizioni di normossia tissutale, il legame dello NO è sfavorito e la respirazione cellulare procede normalmente; al contrario, in condizioni di ipossia, il legame dello NO è favorito, l'enzima riduce la sua attività e di conseguenza la respirazione rallenta.

### **3.3 S-nitrosilazione delle proteine**

Molte delle azioni biologiche mediate dallo NO dipendono dalla sua capacità di interagire con specifici residui aminoacidici di diverse proteine. Alcune di queste modificazioni sono irreversibili e potenzialmente nocive, quali ad esempio la nitratura della tirosina o l'ossidazione della metionina; mentre altre, come la S-nitrosilazione di residui cisteinici e la nitrosilazione del gruppo eme, sono reversibili e in grado di regolare finemente l'attività della proteina (Hess et al., 2005). Diversi studi recentemente hanno focalizzato l'attenzione su una modificazione post-trasduzionale responsabile della modulazione di importanti risposte biologiche: la S-nitrosilazione. Essa consiste nel legame covalente tra lo NO e il gruppo tiolico di una cisteina con formazione di un nitrosotiole (Myers et al., 1990; Stamler et al., 1992) ed è in grado di regolare diversi processi che coinvolgono ad esempio i recettori accoppiati a proteine G (Whalen et al., 2007), la Hsp90 (Martinez-Ruiz et al., 2005), le cascate mitocondriali pro- e anti- apoptotiche (Choi et al., 2002).

#### 4. SINTESI NOS-INDIPENDENTE DELLO NO

Negli ultimi anni diverse evidenze sperimentali hanno dimostrato che l'anione  $\text{NO}_2^-$ , presente in quantità importanti nel sangue e nei tessuti, rappresenta la più grande riserva intravascolare e tissutale di NO.

La riduzione dei nitriti a NO in ambiente acido o riducente è stato mostrato avvenire nello stomaco (Lundberg et al., 1994), nella cavità orale (Duncan et al., 1995), nelle urine (Lundberg et al., 1997), sulla superficie della cute (Weller et al., 1996), oltre che nel cuore ischemico di ratto (Zweier et al., 1995).

Lo NO, infatti, può essere generato anche in maniera NOS-indipendente sia per riduzione enzimatica che per disproporzionazione o riduzione acida dei nitriti (Cosby et al., 2003), ad opera di proteine che mostrano capacità nitrito-reduttasica come la xantina ossidoreduttasi, il citocromo P-450, la deossiemoglobina e, come recentemente evidenziato, anche la eNOS (Gautier et al., 2006) (*fig. 1*).

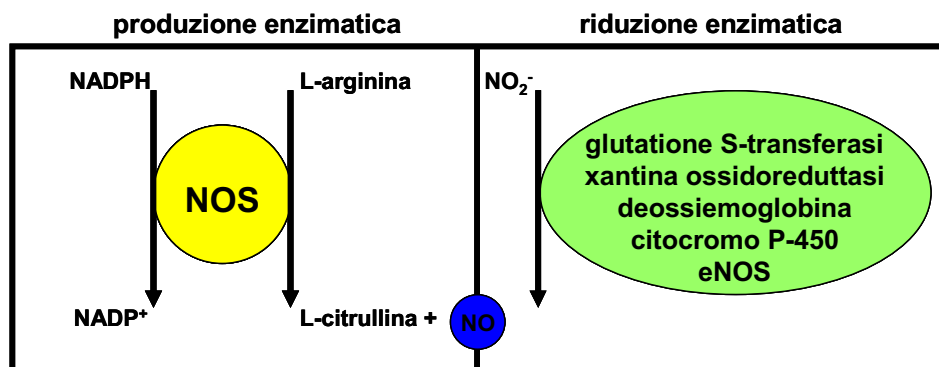


Figura 1. Meccanismi di produzione dello NO.

Questo meccanismo alternativo di produzione dello NO è stato riscontrato soprattutto in condizioni di ipossia e di acidosi quando l'attività delle NOS è fortemente ridotta (Duranski et al., 2005; Webb et al., 2004). È stato osservato che la formazione di NO a partire dai nitriti, nei tessuti biologici, può aumentare fino a circa 100 volte al variare del pH (Benjamin et al., 1994).

Tuttavia, recentemente, alcuni autori hanno riportato che il nitrito stesso è una molecola segnale anche in condizioni fisiologiche: esso agisce da modulatore della stimolazione della sGC, dell'attività del citocromo P-450 e dell'espressione di due importanti proteine, heat shock protein 70 (Hsp 70) e heme oxygenase-1 (HO-1) (Bryan et al., 2005).

## **5. ANIONE NITRITO**

L'anione nitrito è normalmente presente nella maggior parte degli organismi e deriva sia dall'ossidazione dello NO in condizioni aerobiche (Lauer et al., 2001) che dalla dieta, essendo contenuto in diverse verdure e bevande (Weitzberg & Lundberg, 1998); inoltre, può derivare anche dall'anione nitrato anch'esso prodotto dall'ossidazione dello NO oppure proveniente dalla dieta (Spiegelhalder et al., 1976).

La lunga e ricca storia degli effetti benefici e fisiologici dei nitriti è riportata nella *tabella 2*.

#### STORIA DEI NITRITI

Per la prima volta si riporta che...

- 5000 anni fa: i nitriti vengono impiegati nella conservazione dei cibi
- Ottavo secolo: i nitriti vengono impiegati nei disordini cardiovascolari
- 1865: è descritta la reazione tra ossido nitrico ed emoglobina
- 1867: i nitriti vengono impiegati nel trattamento dell'angina come vasodilatatori
- 1899: i nitriti piuttosto che i nitrati conferiscono il colore rosso alla carne
- 1916: gli uomini producono nitrati
- 1928: vengono descritti gli effetti antimicrobici dei nitriti
- 1937: è descritta la reazione tra nitriti e deossiemoglobina
- 1970: i nitriti sono efficaci contro il batterio che causa il botulismo
- 1977: l'ossido nitrico attiva la guanilato ciclastasi
- 1981: i mammiferi sintetizzano i nitrati
- 1986: il fattore EDRF è considerato essere l'ossido nitrico
- 1987: l'efficacia dei nitrovasodilatatori dipende dalla loro capacità di liberare NO
- 1987: i macrofagi producono nitriti e nitrati
- 1988: le cellule neuronali producono NO

**Tabella 2.**

### **5.1 I nitriti: un'importante risorsa fisiologica di NO**

Negli ultimi anni la conversione dei nitriti in NO è stata analizzata con grande interesse e sono stati anche chiariti alcuni meccanismi.

Doyle e collaboratori (1981) hanno analizzato il ruolo di riduzione del nitrito della emoglobina deossigenata: lo ione nitrito reagisce con l'emoglobina (che contiene ione ferroso) e forma metaemoglobina (che contiene ione ferrico) e NO, con conseguente azione vasodilatante. Poiché la metaemoglobina ha un'affinità molto bassa per l'ossigeno, dosi elevate di nitriti potrebbero risultare pericolose, ma solitamente i livelli plasmatici di nitrito sono troppo bassi per causare una significativa metaemoglobinemia (Gladwin et al., 2004).

La deossiemoglobina non è, però, la sola deputata a tale conversione. La produzione di NO a partire da  $\text{NO}_2^-$  si ha in presenza di

altre proteine o di altri enzimi, quali la glutatione-S-transferasi (Hill et al., 1992), la xantina ossidoreduttasi (Millar et al., 1998), il citocromo P-450 (Kozlov et al., 2003).

Questo meccanismo alternativo di sintesi dello NO risulta particolarmente importante in condizioni ischemiche, in cui la biosintesi NOS-dipendente viene meno (Webb et al., 2004). La produzione di NO, infatti, a partire dalla L-arginina operata dalle NOS dipende dall'ossigeno, che viene rapidamente esaurito nell'ischemia. In particolare, le condizioni ischemiche di bassa tensione di ossigeno e di basso pH sono indispensabili perché avvenga la riduzione del nitrito ad opera delle xantine ossidoreduttasi (Zhang et al., 1998). Le xantine ossidoreduttasi mantengono dunque i livelli dello NO in situazioni in cui l'attività delle NOS risulta essere compromessa, come ad esempio nell'infarto del miocardio o in caso di infiammazione cardiovascolare (Li et al., 2001).

Altri studi suggeriscono, inoltre, che la riduzione del nitrito a NO possa derivare da una semplice reazione di acidificazione (Ferrari et al., 1996).

Infine, Gautier e collaboratori (2006) hanno riscontrato tale capacità riduttiva anche nella eNOS in assenza di ossigeno. Mentre in condizioni di normossia la eNOS produce NO a partire dalla L-arginina, in condizioni di anossia l'enzima è ancora capace di produrre NO, ma attraverso un diverso meccanismo: attraverso la riduzione dell'anione  $\text{NO}_2^-$ .

Il nitrito può essere quindi considerato un critico “tampone di ipossia” capace di fornire NO in maniera indipendente dalla presenza di ossigeno, dunque una molecola chiave nella vasodilatazione ipossica, nella respirazione mitocondriale ipossica e nel danno da ischemia-riperfusion.

Lo NO protegge dai danni ischemici in quanto migliora la perfusione sanguigna inducendo vasodilatazione e stimolando la formazione di vasi collaterali (Luque Contreras et al., 2006). Inoltre rallenta la respirazione cellulare attraverso la nitrosilazione del complesso I della catena di trasporto degli elettroni e riduce la produzione di specie reattive dell'ossigeno prodotte in condizioni ischemiche (Shiva et al., 2007). Tuttavia, se da un lato lo NO può essere considerato come un “farmaco” in grado di stimolare il processo di rivascolarizzazione e la conseguente protezione dei tessuti dai danni ischemici, dall'altro lato elevate concentrazioni di NO possono risultare tossiche in quanto il suo potenziale terapeutico dipende sia dal contesto che dalla dose.

E' alla luce di tali evidenze che l'azione protettiva dei nitriti potrebbe essere ancora più efficace di quella direttamente mediata dallo NO. Tali anioni, infatti, vengono ridotti a NO in condizioni ischemiche, mentre vengono convertiti in nitrati in tessuti ben ossigenati (Kumar et al., 2008). In altre parole, i nitriti agiscono come un “pro-farmaco sito-selettivo” in grado di generare NO prevalentemente in condizioni ischemiche con conseguente rivascolarizzazione e protezione cellulare.

Da diversi studi emerge il potenziale ruolo dei nitriti nel trattamento delle patologie caratterizzate da ischemia e disfunzioni emodinamiche, quali ipertensione polmonare neonatale, emorragie associate a vasospasmo, danno da ischemia-riperfusion.

### **5.2I nitriti: un'importante molecola segnale**

L'azione dei nitriti non è soltanto confinata al ruolo che svolgono in condizioni ischemiche ma tali anioni sono implicati in diversi meccanismi anche in condizioni fisiologiche. Bryan e collaboratori (2005) hanno infatti riportato che i nitriti rappresentano essi stessi un'importante molecola segnale in condizioni fisiologiche. In particolare, i nitriti aumentano i livelli di cGMP, inibiscono l'attività del citocromo P450 e modulano l'espressione di due importanti proteine, heat shock protein 70 (Hsp 70) e heme oxygenase-1 (HO-1). Tali effetti sono stati osservati in diversi tessuti *in vivo*. Inoltre diverse azioni mediate dai nitriti non sembrano essere dovute alla loro conversione a NO, ma piuttosto sembrano conseguenti alla formazione di specie S/N-nitroso e ferro-nitrosil-eme a partire da enzimi contenenti gruppi tiolici o gruppi eme.

### **5.3I nitriti come indice dell'attività delle NOS**

I livelli plasmatici dei nitriti sono ben conservati nelle varie specie di mammifero, incluso l'uomo, in un range che varia tra 150 e 600 nM (Kleinbongard et al., 2003). Circa l'80-90% dei nitriti nel plasma deriva dal metabolismo dello NO prodotto dalla eNOS, perciò i nitriti possono essere

considerati un valido indice dell'attività della eNOS e, contemporaneamente, un valido bio-marker della funzionalità endoteliale. I livelli di nitrito nel plasma rispecchiano dunque i cambiamenti nell'attività della eNOS, nonché la presenza di disfunzioni endoteliali in soggetti affetti da patologie cardiovascolari (Lauer et al., 2001).

## **6. EFFETTI CARDIACI DEI NITRITI**

In condizioni di ischemia o ipossia si crea l'ambiente acido o riducente indispensabile per la produzione NOS-indipendente di NO. Lo NO è un importante mediatore paracrino del sistema cardiovascolare che a basse concentrazioni modula diverse risposte. Sono stati osservati sia effetti inotropi negativi che positivi ad opera dello NO, a seconda del modello preso in esame e del tipo e della dose del donatore di NO usati (Sandrasegarane & Diamond, 1999; Sarkar et al., 2000; Sarkar et al., 2001; Paolucci et al., 2003). Inoltre, lo NO ha un ruolo chiave nella regolazione del tono vasale sia del circolo coronarico (Kelm & Schrader, 1990) che del circolo sistemico (Lauer et al., 2001).

Zweier e collaboratori (1995) dimostrarono che, nel cuore isolato e perfuso di ratto, la produzione di NO aumenta di circa 10 volte dopo 30 minuti di ischemia globale, un incremento che è solo parzialmente inibito dallo L-NAME.

I meccanismi osservati in tali studi riflettono probabilmente un fenomeno più generale che riguarda diversi tessuti in condizioni di ischemia



o scarsa perfusione e che potrebbe costituire la principale fonte di NO. Ad esempio, Hunter e collaboratori (2004) dimostrarono che dopo inalazione di nitrito di sodio da parte di agnelli neonati affetti da ipertensione polmonare ipossica, si rileva un importante miglioramento dell'ipertensione, associato all'immediata presenza di NO nel gas espirato. Inoltre, è stato anche rilevato che la vasodilatazione polmonare provocata dal nitrito inalato è deossiemoglobina- e pH-dipendente. Dati quest'ultimi che confermano il meccanismo di azione del nitrito sopra descritto.

Nel cuore isolato e perfuso di ratto si genera NO a partire dall'anione  $\text{NO}_2^-$  attraverso una reazione che dipende dall'attività della xantina ossidoreduttasi (Zhang et al., 1998; Li et al., 2001). La conferma di tale meccanismo si è avuta mediante studi condotti con l'allopurinolo, un inibitore della xantina ossidoreduttasi: è stato osservato che, inibendo l'enzima, si ha anche la soppressione della produzione di NO (Webb et al., 2004).

Sebbene la xantina ossidoreduttasi sia nota per la riduzione dell'ossigeno molecolare ad anione superossido ( $\text{O}_2^-$ ), a basse tensioni di ossigeno (Kayyali et al., 2001) e a bassi valori di pH (Godber et al., 2000), essa è anche capace di ridurre i nitriti a NO. Tale trasformazione richiede abbondanti quantità di superossido dismutasi (SOD) per catturare l' $\text{O}_2^-$  che altrimenti reagisce rapidamente con lo NO formando  $\text{ONOO}^-$ ,  $\text{NO}_2$  e/o  $\text{N}_2\text{O}_3$ .

La produzione di NO a partire dai nitriti è associata a miglioramenti nel recupero della funzionalità cardiaca (Bolli, 2001) e dunque a un'azione

cardioprotettiva che viene completamente bloccata da scavenger dello NO, quali mioglobina (Brunori, 2001) o carboxy-PTIO (Webb et al., 2004).

Diverse evidenze sperimentali hanno chiarito dunque un importante ruolo dei nitriti: la loro conversione a NO protegge il miocardio dai danni da ischemia-riperfusione. E' vero però che la consapevolezza che coesistono sia NO-sintasi sia NO<sub>2</sub><sup>-</sup>-reduttasi suggerisce che la via dei nitriti potrebbe anche essere coinvolta in importanti funzioni distinte da quelle dello NO. Ciò fa ipotizzare, inoltre, che le azioni tessuto-protettive dei nitriti potrebbero non essere limitate soltanto ad eventi ischemici, ma verificarsi anche in condizioni fisiologiche.

## ***MATERIALI E METODI***

## 1. PROCEDURA SPERIMENTALE

I modelli sperimentali utilizzati in questo lavoro sono stati il cuore isolato e perfuso di anguilla, icefish e rana secondo la metodica “working” e il cuore isolato e perfuso di ratto secondo la metodica *Langendorff*.

Tali preparati permettono di ottenere condizioni altamente standardizzate che riflettono le caratteristiche emodinamiche dell'attività cardiaca dell'animale *in vivo* e risultano ottimali per effettuare studi fisiologici scevri da artefatti sperimentali.

## 2. GLI ANIMALI

Gli esperimenti sono stati condotti utilizzando esemplari di *Anguilla anguilla*, *Chionodraco hamatus* (icefish), *Rana esculenta* e ratti Wistar del peso corporeo rispettivamente di 90-120 g, 320-370 g, 15-25 g, 180-250 g.

Le anguille, fornite da un vivaio locale, sono state stabulate a digiuno in vasche contenenti acqua dolce continuamente filtrata e areata, ad una temperatura compresa tra 18 e 20°C.

Gli icefish, catturati durante la XX Spedizione Italiana in Antartide (dicembre 2004-febbraio 2005), sono stati mantenuti per almeno 5 giorni a digiuno in vasche contenenti acqua di mare continuamente filtrata e areata, ad una temperatura compresa tra 0 e 2°C.

Le rane, provenienti da un allevamento locale, sono state tenute a digiuno in un acquaterraio a temperatura ambiente (18-20°C) dove sono state stabulate prima di essere sacrificate.

I ratti, forniti dalla Harlan Laboratories s.r.l. (Udine, Italia), sono stati mantenuti in locali climatizzati ed illuminati artificialmente per cicli di 12 ore consecutivi, sono stati alimentati *ad libitum*. I ratti sono stati stabulati per almeno quattro giorni prima di essere utilizzati (per permettere loro il recupero dallo stress conseguente al trasporto) e sono stati sacrificati entro 20 giorni dall'arrivo nello stabulario.

### **3. DISSEZIONE ED ISOLAMENTO DEL CUORE**

**Anguilla e icefish.** Prima di procedere alla dissezione, anguilla e icefish sono anestetizzati per circa 15 minuti in benzocaina e despinalizzati mediante recisione della colonna vertebrale.

L'isolamento del cuore è effettuato con l'uso di uno stereomicroscopio ed il preparato è continuamente idratato con soluzione fisiologica.

Dopo aver aperto ventralmente l'animale lungo la linea longitudinale mediana, all'altezza delle pinne pettorali, sono asportati la muscolatura ventrale, il pericardio e i legamenti che uniscono quest'ultimo al cuore. Vengono quindi effettuati due tagli: il primo in corrispondenza dell'aorta ed il secondo a livello del seno venoso. Il cuore è posto in una capsula di Petri contenente soluzione fisiologica e due cannule fissate temporaneamente; la

cannula di input è inserita nel seno venoso e la cannula di output nell'aorta. Al termine della procedura di isolamento, il cuore è collegato all'apparato di perfusione.

**Rana.** Le rane sono decapitate, despinalizzate per eliminarne i riflessi e fissate su una base di dissezione. L'animale è aperto ventralmente praticando un'incisione a T sulla cute e tagliando i muscoli sottostanti e lo sterno. Inizialmente viene tolto il pericardio e viene legato il tronco aortico destro per poi tagliarlo a monte del nodo; spostando lateralmente il cuore vengono messe in evidenza le precave destra e sinistra e quindi legate e tagliate; la vena polmonare viene legata e tagliata prima della biforcazione. Viene praticata un'incisione a becco di flauto sul tronco aortico sinistro che è dunque legato ad una cannula di polietilene riempita di eparina. Sollevato il ventricolo, viene separato il cuore dai legamenti e viene messo in evidenza il seno venoso che rappresenta la via di ingresso. Dopo aver praticato un'incisione, nel vaso viene inserita la cannula riempita di Ringer. Il cuore così isolato è collegato all'apparato di perfusione.

**Ratto.** I ratti vengono anestetizzati mediante iniezione intraperitoneale di carbammato di etile. L'isolamento si effettua mediante apertura del torace attraverso un'incisione bilaterale lungo il margine inferiore dalle prime alle ultime costole così da rimuovere la cute e i muscoli sottostanti.

Dopo l'incisione dell'aorta, delle vene cave e dei vasi polmonari, si procede al vero e proprio espianto del cuore che viene immediatamente

immerso in un beker contenente la soluzione fisiologica mantenuta a 4°C allo scopo di limitare i danni ischemici (Hearse & Sutherland, 2000). Il cuore viene rapidamente incannulato attraverso l'aorta per dare l'avvio alla perfusione retrograda a flusso costante ( $10-13.5 \text{ ml min}^{-1} \text{ g}^{-1}$  di cuore fresco). Il perfusato viene rilasciato in una direzione retrograda poiché le valvole aortiche sono forzatamente chiuse e il fluido di perfusione è diretto all'interno dei vasi coronarici per irrorare l'intera massa ventricolare, l'atrio di destra e defluire attraverso il seno coronarico. Di seguito viene praticato un foro all'apice del ventricolo sinistro per evitare l'accumulo di liquido ed un altro a livello dell'arteria polmonare per garantire un adeguato drenaggio del liquido in uscita dal cuore (infatti accidentalmente potrebbe capitare di legare, insieme all'aorta, anche l'arteria polmonare). Il tempo che intercorre tra l'espanto e l'inizio della riperfusione è di 30 secondi. Dal momento in cui si avvia la riperfusione, la funzione contrattile e il ritmo cardiaco si ripristineranno in pochi secondi tuttavia dovranno passare all'incirca 10 minuti prima che il cuore si stabilizzi definitivamente.

L'attività contrattile del cuore viene misurata mediante l'utilizzo di un palloncino di lattice inserito nel ventricolo sinistro e collegato ad un trasduttore di pressione (Modello BLPR, cod. 18700). Per agevolare l'inserimento del palloncino attraverso la valvola mitrale nella cavità ventricolare viene rimosso l'atrio sinistro. Una volta posizionato all'interno del ventricolo, il palloncino viene gonfiato con acqua distillata mediante una microsiringa (Hamilton da 250  $\mu\text{L}$ ) per regolare il volume al suo interno. I

palloncini sono utilizzabili solo se ad un volume di 80-100  $\mu$ L generano una pressione compresa tra +2 mmHg e -2 mmHg e comunque inferiore a 3 mmHg (Klebanov et al., 1997). Il volume del palloncino viene aumentato con incrementi graduali di 20  $\mu$ L fino a quando il valore della pressione diastolica risulti intorno a 5 mmHg. Questo valore corrisponde al lavoro isovolumetrico del cuore (Bartfay et al., 1999). Volumi eccessivi comporterebbero un allungamento tale delle fibre muscolari per cui, non venendo rispettata la legge di Starling, si causerebbe la compressione del tessuto cardiaco inducendo ischemia e rendendo instabile il preparato con un'aumentata tendenza a sviluppare aritmie (Sutherland & Hearse, 2000) .

Una volta completati questi passaggi, si pone il cuore in una camera mantenuta a temperatura costante (37°C) mediante l'uso di un bagno termostato a circolazione interna.

#### **4. APPARATO DI PERFUSIONE**

L'apparato di perfusione di anguilla, icefish e rana (*fig. 2A-B*) è costituito da una pompa peristaltica, da una camera di perfusione e da due serbatoi, uno d'ingresso e uno d'uscita. Il Ringer o soluzione di perfusione è contenuto in un serbatoio esterno da cui è prelevato dalla pompa peristaltica e, attraverso il serbatoio di ingresso, raggiunge la cannula inserita nel seno venoso. Il perfusato, pompato dal cuore, si raccoglie nel serbatoio d'uscita.



La camera di perfusione, rappresentata da un contenitore in plexiglas dove viene alloggiato il cuore, viene riempita di soluzione fisiologica in modo da coprire interamente il cuore, al fine di evitarne la disidratazione.

I valori medi delle pressioni di ingresso e di uscita, precarico e postcarico, sono misurati per mezzo di due trasduttori di pressione collegati con rispettivi apparecchi di registrazione.

L'apparato usato per perfondere il cuore attraverso la tecnica del *Langendorff* (fig. 2C) consta di un serbatoio contenente la soluzione di perfusione, una trappola per bolle, una pompa peristaltica, un bagno termoregolatore a circolazione esterna e un sistema di acquisizione dati con interfaccia McLab.

Il liquido di perfusione viene prelevato dal serbatoio tramite una pompa peristaltica, viene spinto attraverso una serie di tubi nella trappola per bolle che ha lo scopo di evitare la formazione di emboli e giunge al cuore attraverso una cannula inserita nell'aorta.

L'intero sistema di perfusione viene mantenuto alla temperatura di 37°C mediante diversi tubi collegati ad un bagno termostato che garantiscono il ricircolo d'acqua calda attraverso la camicia esterna del serbatoio, della camera contenente il cuore e della trappola per bolle.

La pressione del ventricolo sinistro e la pressione coronarica vengono registrate tramite l'uso di due trasduttori (Model BLPR, cod.18700) collegati al sistema di acquisizione dati McLab, a sua volta collegato ad un computer (Pentium III, Hewelett Packard Brio).

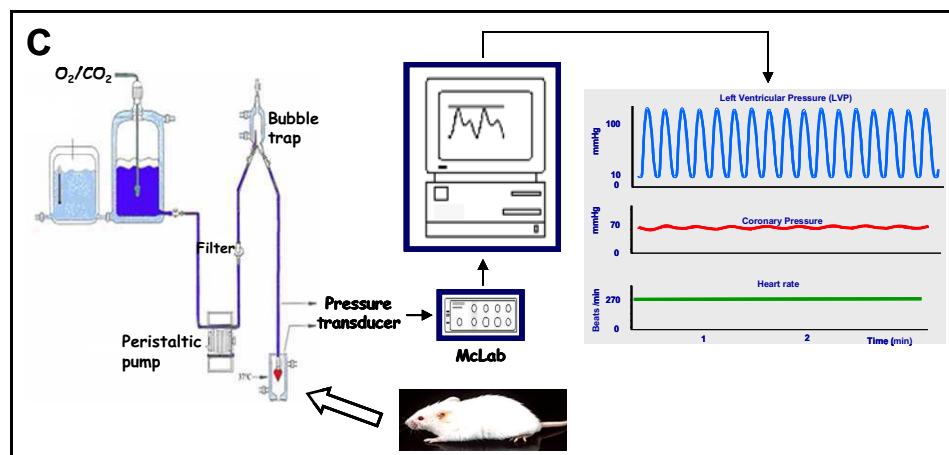
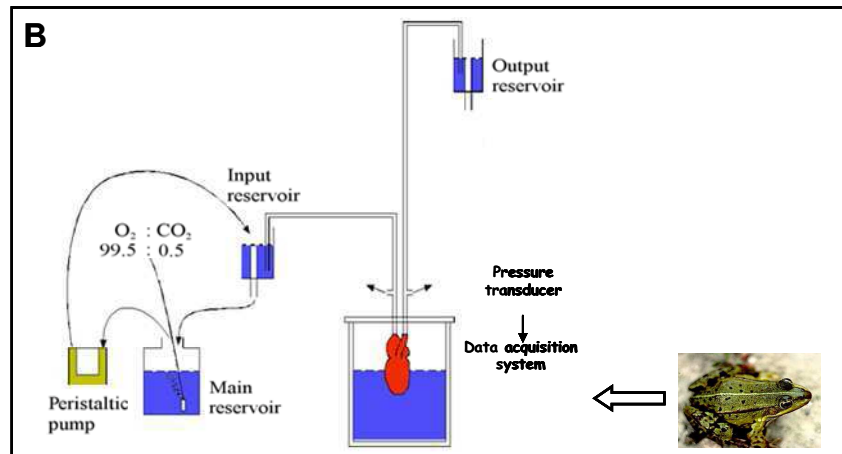
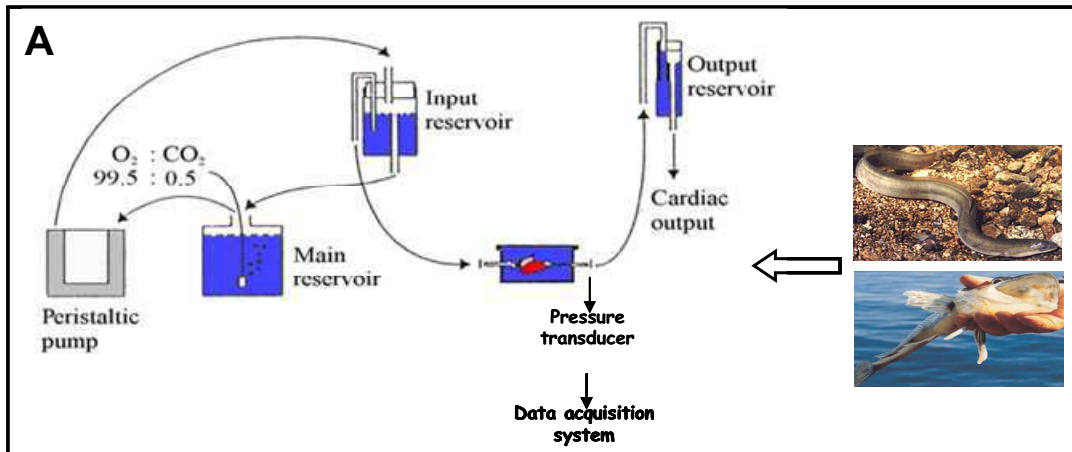


Figura 2. Apparato di perfusione dei pesci (A); della rana (B); del ratto (C).

## 5. COMPOSIZIONE DEL PERFUSATO

La soluzione Ringer utilizzata nei pesci ha la seguente composizione:

NaCl	6.68 $\text{gl}^{-1}$
KCl	0.15 $\text{gl}^{-1}$
$\text{KH}_2\text{PO}_4$	0.05 $\text{gl}^{-1}$
$\text{MgSO}_4$	0.35 $\text{gl}^{-1}$
$(\text{NH}_4)_2\text{SO}_4$	0.05 $\text{gl}^{-1}$
$\text{Na}_2\text{HPO}_4$	0.227 $\text{gl}^{-1}$
Glucosio	1 $\text{gl}^{-1}$
$\text{CaCl}_2$	0.14 $\text{gl}^{-1}$

Il Ringer è stato gassificato con una miscela costituita da  $\text{O}_2$  e  $\text{CO}_2$  in percentuale del 99.5:0.5% e stabilizzato a un valore di pH compreso tra 7.7-7.9 con circa 1  $\text{gl}^{-1}$  di  $\text{NaHCO}_3$ .

La soluzione Ringer utilizzata nella rana ha la seguente composizione:

NaCl	6.6 $\text{gl}^{-1}$
KCl	0.186 $\text{gl}^{-1}$
$\text{CaCl}_2$	0.11 $\text{gl}^{-1}$
$\text{Na}_2\text{HPO}_4$	0.475 $\text{gl}^{-1}$
$\text{NaH}_2\text{PO}_4$	0.1 $\text{gl}^{-1}$
Glucosio	1.009 $\text{gl}^{-1}$

Il Ringer è stato fatto aerare per circa un'ora con una pompa ad aria ed il pH è stato regolato intorno ad un valore di 7.2.

Il liquido di perfusione utilizzato nel ratto è costituito da un tampone bicarbonato modificato di Krebs-Henseleit (Legssyer et al., 1997; Doring, 1990) avente la seguente composizione:

NaCl	113 mM
KCl	4.7 mM
MgSO <sub>4</sub> 7H <sub>2</sub> O	1.2 mM
CaCl <sub>2</sub> 2H <sub>2</sub> O	1.8 mM
NaHCO <sub>3</sub>	25 mM
KH <sub>2</sub> PO <sub>4</sub>	1.2 mM
Sodio Piruvato	5 mM
Glucosio	11 mM
Mannitolo	1.1 mM

Una volta preparato, il liquido di perfusione viene filtrato attraverso una membrana di cellulosa da 0.8 micron al fine di rimuovere le eventuali impurità presenti e viene mantenuto ad un pH pari a 7.4 attraverso la continua ossigenazione con una miscela di 95% O<sub>2</sub> / 5% CO<sub>2</sub>. In tal modo il cuore viene ossigenato e si evita anche la precipitazione dei sali di calcio.

## **6. MISURA E CALCOLO DEI PARAMETRI CARDIACI**

I valori di pressioni di ingresso e di uscita nei pesci e nella rana sono stati rilevati mediante il trasduttore di pressione a sua volta collegato con un sistema di acquisizione dati con interfaccia McLab. Il precarico e il postcarico sono stati riferiti ai livelli di perfusato nelle camere di perfusione ed i valori pressori rilevati sono stati corretti per la resistenza delle cannule ed espressi in kPa.

La frequenza cardiaca (HR, batt/min) è stata ricavata dai tracciati di pressione.

La gittata cardiaca (CO, ml/min/Kg) è stata determinata pesando il perfusato raccolto in un minuto dal serbatoio di uscita. I valori del peso sono stati successivamente corretti tenendo conto della densità del liquido di perfusione e del peso dell'animale espresso in Kg.

La pressione massima (Pmax) è stata ricavata dai tracciati di pressione, misurando la distanza in mm che intercorre tra lo zero e il picco massimo e moltiplicando il valore ottenuto per il fattore di conversione dello strumento.

La pressione minima (Pmin) è stata ricavata dai tracciati di pressione, misurando la distanza in mm che intercorre tra lo zero e il picco minimo e moltiplicando il valore ottenuto per il fattore di conversione dello strumento.

La pressione media (Pmedia) è stata ottenuta dai seguenti calcoli:  
 $2/3$  pressione minima +  $1/3$  pressione massima.

Il volume sistolico (Stroke Volume = CO/HR, ml/Kg) è stato usato come indice della performance ventricolare; cambiamenti nello SV sono stati considerati effetti inotropi.

Il lavoro cardiaco [Stroke Work = (postcarico - precarico) x SV / Peso Ventricolare, mJ/g] è stato utilizzato come indice di funzionalità sistolica.

I parametri emodinamici del ratto vengono calcolati in tempo reale e salvati su un foglio di supporto "Data Pad". Alla fine della registrazione è possibile trasferire i dati immagazzinati sul Data Pad su un file Microsoft Excel per l'elaborazione statistica.

Possono essere analizzati i seguenti parametri emodinamici:

1) parametri relativi all'attività sistolica:

- LVP (Left Ventricular Pressure, mmHg), rappresenta un indice della pressione sviluppata dal ventricolo sinistro in condizioni di lavoro isovolumetrico;
- $+(LVdP/dt)_{max}$  (mmHg x s<sup>-1</sup>), definito Vmax, indica la massima velocità di contrazione ventricolare;
- TTP (Time To Peak, ms), rappresenta il tempo necessario a raggiungere il picco di contrazione sistolica isovolumetrica;
- HR (Heart Rate, battiti x min<sup>-1</sup>), rappresenta la frequenza cardiaca;
- RPP (Rate Pressure Product, mmHg x battiti x min<sup>-1</sup>), pari al prodotto tra LVP ed HR, rappresenta un indice del lavoro cardiaco;

2) parametri relativi al recupero diastolico:

- LVEDP (Left Ventricular End Diastolic Pressure, mmHg x s<sup>-1</sup>), è la pressione telediastolica sviluppata dal ventricolo sinistro;
- $-(LVdP/dt)_{max}$  (mmHg x s<sup>-1</sup>), definita V<sub>min</sub>, indica la massima velocità di rilassamento ventricolare;
- HTR (Half Time Relaxation, ms), indica la metà del tempo necessario al ventricolo sinistro per il completo recupero diastolico;
- T/t è dato dal rapporto tra V<sub>max</sub> e V<sub>min</sub>;

3) parametri relativi alla funzionalità coronarica:

- CP (Coronary Pressure, mmHg), è la pressione di perfusione coronarica.

## **7. CONDIZIONI BASALI DI PERFUSIONE**

La perfusione è avviata, in condizioni basali, per un periodo di circa 15-20 minuti, necessari per permettere la stabilizzazione della performance cardiaca. I cuori che non si stabilizzano entro 15-20 minuti dall'inizio della perfusione sono scartati. I parametri cardiaci sono registrati per tutto il periodo della perfusione.

L'elettrostimolazione dei cuori è utilizzata al fine di poter distinguere l'azione inotropica (variazione della forza di contrazione) da quella cronotropica (variazione della frequenza cardiaca).

## **8. COMPOSTI CHIMICI**

Negli esperimenti condotti sono stati utilizzati: nitrito di sodio; L-arginine; N-monomethyl-L-arginine (L-NMMA); 3-morpholinosydnonimine (SIN-1); superoxide dismutase (SOD); N-iminoethyl-L-ornithine (L-NIO); 2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO); 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ); KT5823.

Tutte le sostanze sono state preparate sottoforma di soluzione madre in acqua bi distillata, eccetto lo ODQ che è stato preparato in etanolo. Le soluzioni madre sono state successivamente diluite in soluzione di perfusione in modo da ottenere le concentrazioni utilizzate nei singoli esperimenti.

Tutti i composti sono stati forniti dalla Sigma Chemical Company (St. Louis, MO, USA).

Gli esperimenti con sostanze fotosensibili, quale KT5823, sono stati effettuati utilizzando un apparato modificato in modo da proteggere la sostanza dalla luce ed evitarne la degradazione.

## **9. PROTOCOLLO DI PERFUSIONE**

Dopo aver stabilizzato il preparato alle condizioni basali di perfusione, sono state testate dosi crescenti di nitriti. Gli effetti sui parametri emodinamici sono stati valutati dopo 10 minuti dall'inizio dell'applicazione della sostanza. Ogni cuore ha rappresentato il controllo di se stesso.



Per valutare gli effetti dei nitriti in presenza di altre sostanze, il preparato cardiaco è stato perfuso prima con solo nitrito e successivamente con nitrito in presenza della sostanza.

## **10. PROTOCOLLO DI OMOGENIZZAZIONE**

I cuori vengono omogenizzati mediante un polytron, in ghiaccio in RIPA buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1% Igepal CA 630, 0.5% Sodium Deoxycholate, 1 mM EDTA, 0.1% SDS, 200 mM Sodium Orthovanadate, Protease Inhibitor Cocktail). L'omogenato viene centrifugato a 4°C per 40 minuti a 13.000 g. Il sovrantante contenente la frazione citosolica viene raccolto, mentre il pellet contenente la frazione di membrana viene risospeso. La lettura proteica viene effettuata utilizzando il Bradford.

## **11. BIOTIN SWITCH E WESTERN BLOT**

Al fine di rilevare le proteine S-nitrosilate è stato utilizzato il metodo "Biotin Switch" essenzialmente come riportato da Jaffrey e Snyder nel 2001.

Tale saggio prevede come primo step il blocco dei gruppi SH, come secondo step la riduzione dei gruppi SNO a gruppi SH e come terzo step il legame della biotina a tali gruppi SH. Le proteine così "biotinilate" vengono sottoposte ad un comune Western Blot: in seguito alla corsa elettroforetica e al trasferimento, le membrane vengono incubate con la streptavidina e dalla rilevazione del complesso biotina-streptavidina si ha dunque la

possibilità di analizzare i livelli di nitrosilazione delle proteine di cuori controllo e cuori trattati.

I cuori di anguilla, rana e ratto perfusi sono stati istantaneamente congelati in azoto liquido e al momento opportuno omogenati e sottoposti al cosiddetto “Biotin Switch Assay” (Jaffrey et al., 2001; Jaffrey & Snyder, 2001). Il campione contenente 0.5 mg/ml di proteine viene prima trattato con un “blocking buffer” (225 mM Hepes, pH 7.7, 0.9 mM EDTA, 0.09 mM neocuproine, 2.5% SDS, 20 mM MMTS) a 50°C per 20 minuti perché vengano bloccati i gruppi SH liberi, poi viene precipitato con acetone freddo, centrifugato a 4°C per 5 minuti a 2000 g e risospeso in “hens buffer” (250 mM Hepes, pH 7.7, 1 mM EDTA, 0.1 mM neocuproine, 1% SDS). Tutte queste operazioni vengono condotte al buio. Successivamente i campioni vengono incubati per 1 ora a temperatura ambiente con una soluzione contenente acido ascorbico e biotina: l'acido ascorbico riduce i gruppi SNO in gruppi SH liberi a cui si lega dunque la biotina. Le proteine così biotinilate sono nuovamente precipitate con acetone freddo, centrifugate a 4°C per 5 minuti a 2000 g e risospese in “hens buffer”.

Tali proteine biotinilate sono sottoposte a Western Blot: le proteine vengono caricate su gel al 15% e, in seguito alla corsa elettroforetica, vengono trasferite su membrana; la membrana è incubata per 1 ora prima con il latte al 5% e poi con la streptavidina diluita 1:5000. Si procede infine con lo sviluppo con ECL su lastra (*fig. 3*).

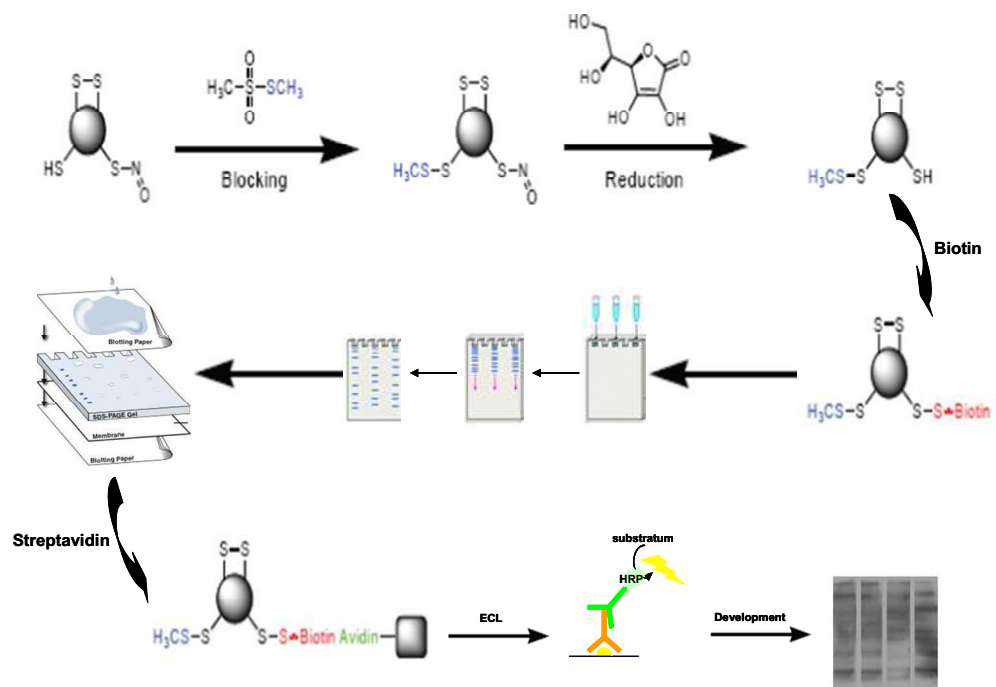


Figura 3. Schema del Biotin Switch Assay e Western Blot.

## 12. ANALISI STATISTICA

Gli effetti sui parametri emodinamici presi in considerazione sono stati espressi come medie  $\pm$  errore standard delle variazioni percentuali dei singoli esperimenti rispetto ai valori di controllo. Poiché ogni cuore rappresenta il controllo di se stesso, le differenze significative tra la condizione test (cioè in presenza della sostanza) e la condizione basale di controllo sono state determinate con l'utilizzo del test di Student per campioni appaiati (un valore di  $p < 0.05$  è considerato significativo \*; un valore di  $p < 0.01$  è considerato molto significativo \*\*; un valore di  $p < 0.001$  è considerato altamente significativo \*\*\*).

Con il test ANOVA sono state valutate le differenze significative tra i diversi gruppi di esperimenti (un valore di  $p < 0.05$  è considerato significativo

§; un valore di  $p < 0.01$  è considerato molto significativo §§; un valore di  $p < 0.001$  è considerato altamente significativo §§§).

## ***RISULTATI***

## 1. EFFETTI DEI NITRITI SULLA PERFORMANCE CARDIACA IN CONDIZIONI BASALI

In questo lavoro è stato analizzato l'effetto dei nitriti su cuori di anguilla, icefish e rana, isolati e perfusi secondo la metodica "working" messa a punto nei nostri laboratori, e su cuori di ratto, isolati e perfusi secondo la metodica *Langendorff*, in condizioni basali cioè in presenza di adeguata ossigenazione ed in assenza di stimolazione chimica.

Gli esperimenti sono stati condotti su cuori stimolati elettricamente al fine di mantenere stabile la frequenza cardiaca. I parametri cardiaci presentati sono: per anguilla, icefish e rana, il volume sistolico come indice della performance ventricolare e il lavoro cardiaco come indice di funzionalità sistolica; per il ratto, LVP che rappresenta la pressione sviluppata dal ventricolo sinistro in condizioni di lavoro isovolumetrico e  $[(LVdp/dt)_{max}]$  che rappresenta la massima velocità di contrazione muscolare. Cambiamenti di questi parametri sono considerati effetti inotropi.

Tutti questi parametri sono stati rilevati per ciascuna dose dopo dieci minuti dall'inizio del trattamento.

Le curve dose-risposta sono state effettuate esponendo i preparati cardiaci a concentrazioni crescenti di nitriti ( $10^{-7} \div 10^{-4}$  M in anguilla, icefish, rana;  $10^{-10} \div 10^{-4}$  M nel ratto) in condizioni basali. I risultati ottenuti dimostrano che i nitriti incidono potentemente sulla "performance" cardiaca, aumentando lo SV nel cuore di icefish e riducendo lo SV nel cuore di anguilla e di rana e lo LVP nel cuore di ratto; inoltre, in tutte le specie,

l'effetto inotropo è concentrazione-dipendente (*fig. 4*). E' interessante inoltre notare che mentre concentrazioni di nitrito comprese tra 100 nM e 100  $\mu$ M mediano significative risposte in tutte le specie studiate, il cuore di ratto è molto più sensibile ai nitriti: con concentrazioni più basse di 1 nM si ha infatti una significativa riduzione dello LVP.

## **2. EFFETTO INOTROPO DELLO NO DI ORIGINE ENDOGENA**

Poiché diversi studi hanno dimostrato che l'azione dei nitriti è mediata dalla loro conversione in NO, abbiamo confrontato l'effetto inotropo esercitato dai nitriti con quello esercitato dallo NO nei nostri modelli.

Per determinare gli effetti dello NO endogeno, abbiamo trattato i cuori con il substrato fisiologico per le NOS, la L-arginina ( $10^{-7}$  M in anguilla;  $10^{-6}$  M in icefish;  $10^{-7}$  M in rana;  $10^{-8}$  M in ratto): la L-arginina induce un significativo aumento dello SV nell'icefish ed una significativa riduzione dello SV nell'anguilla e nella rana e dello LVP nel ratto. In tutte le specie, l'effetto L-arginina-dipendente viene inibito dallo L-NMMA ( $10^{-5}$  M), un inibitore delle NOS (*fig. 5*).

## **3. EFFETTO INOTROPO DELLO NO DI ORIGINE ESOGENA**

Abbiamo inoltre investigato circa gli effetti di un donatore NOS-indipendente di NO, quale il SIN-1 ( $10^{-7}$  M in anguilla;  $10^{-5}$  M in icefish;  $10^{-7}$  M in rana;  $10^{-10}$  M in ratto), somministrato insieme alla superossido

dismutasi (SOD 10 IU/ml) dal momento che rilascia simultaneamente NO e  $O_2^-$ . Il SIN-1 media un effetto inotropo positivo nel cuore di icefish ed un effetto inotropo negativo nel cuore di anguilla, rana e ratto (*fig. 6*).

#### **4. COINVOLGIMENTO DELLE NOS SULL'EFFETTO CARDIACO DEI NITRITI**

Poiché lo NO (proveniente sia da fonti endogene che esogene) e i nitriti esercitano effetti simili sulla performance cardiaca in tutte le specie prese in esame, abbiamo indagato se l'effetto esercitato dai nitriti coinvolgesse la cascata dell'ossido nitrico.

Innanzitutto abbiamo verificato se il meccanismo d'azione dei nitriti coinvolgesse la formazione NOS-indipendente di NO. A tal fine, i cuori sono stati esposti al trattamento con nitrito ( $10^{-5}$  M in anguilla;  $10^{-5}$  M in icefish;  $10^{-5}$  M in rana;  $10^{-8}$  M in ratto) in assenza e in presenza di un inibitore delle NOS, lo L-NIO ( $10^{-5}$  M). Nell'anguilla e nell'icefish il pretrattamento con l'inibitore abolisce completamente l'effetto sull'inotropismo; mentre nella rana non modifica l'effetto nitrito-dipendente e nel ratto sembra potenziarlo, dato quest'ultimo che può essere giustificato dal fatto che lo L-NIO esercita un effetto inotropo negativo anche se somministrato da solo (*fig. 7*).

#### **5. COINVOLGIMENTO DELLO NO SULL'EFFETTO CARDIACO DEI NITRITI**

Per valutare se l'effetto dei nitriti possa attribuirsi alla loro riduzione a NO, i cuori sono stati trattati con nitrito ( $10^{-5}$  M in anguilla;  $10^{-5}$  M in icefish;



$10^{-5}$  M in rana;  $10^{-8}$  M in ratto) in assenza e in presenza di uno scavenger di NO, lo PTIO ( $10^{-7}$  M). Anche tali esperimenti mostrano un diverso andamento a seconda della specie considerata: nell'anguilla e nell'icefish il pretrattamento con PTIO non determina alcuna modificazione significativa, invece, nella rana abolisce l'effetto nitrito-dipendente e nel ratto lo attenua in modo significativo, dimostrando il coinvolgimento dello NO nel meccanismo d'azione dei nitriti (*fig. 8*).

## **6. COINVOLGIMENTO DELLA sGC SULL'EFFETTO CARDIACO DEI NITRITI**

Per analizzare se l'effetto inotropo negativo nitrito-dipendente coinvolga il pathway cGMP/PKG, abbiamo utilizzato lo ODQ ( $10^{-5}$  M), un potente ossidante e inibitore della guanilato ciclastasi solubile, e il KT5823 ( $10^{-7}$  M), un inibitore della PKG.

Tali esperimenti non sono stati condotti anche sull'icefish per mancanza di campione (non facilmente reperibile).

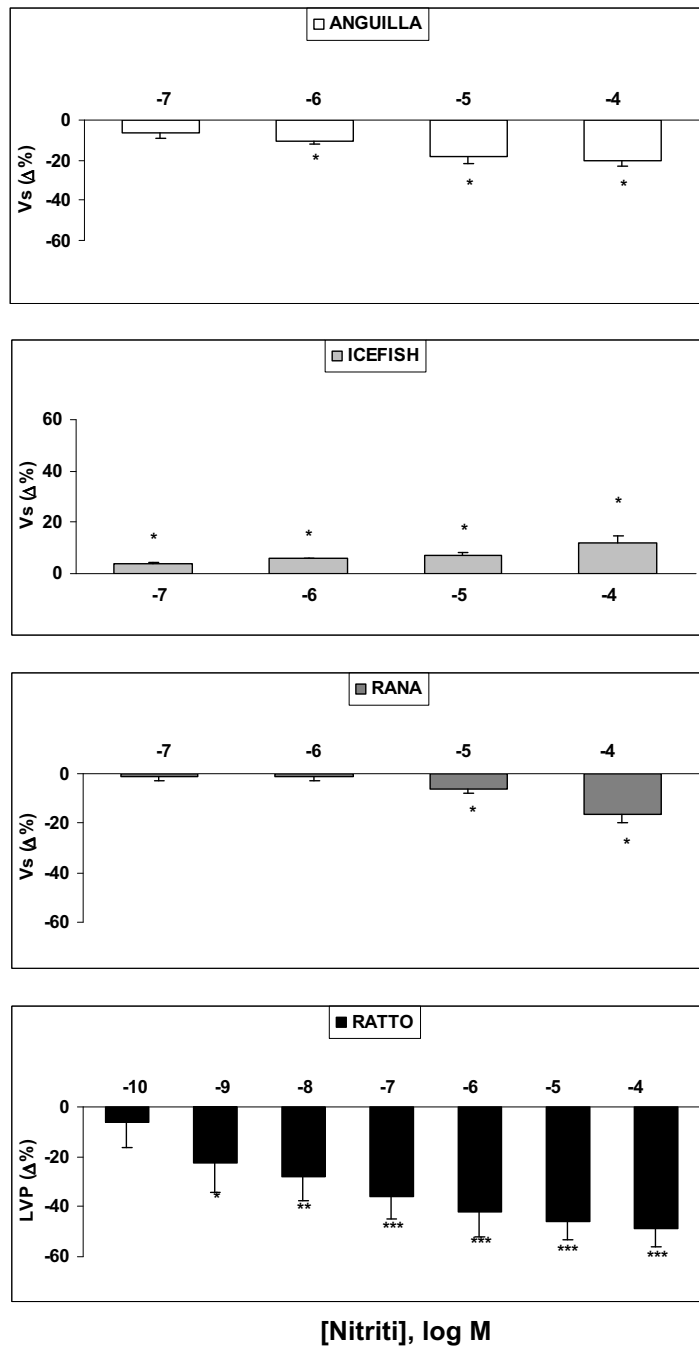
Il trattamento sia con ODQ sia con KT5823 abolisce l'effetto esercitato dai nitriti nelle tre specie prese in esame, suggerendo che l'azione di tali anioni sull'inotropismo sia dipendente dall'attivazione del pathway cGMP/PKG (*fig. 9-10*).

## **7. S-NITROSILAZIONE DELLE PROTEINE CARDIACHE**

Successivamente abbiamo valutato i livelli di nitrosilazione delle proteine cardiache, che sono indice della loro eventuale azione nitrito-

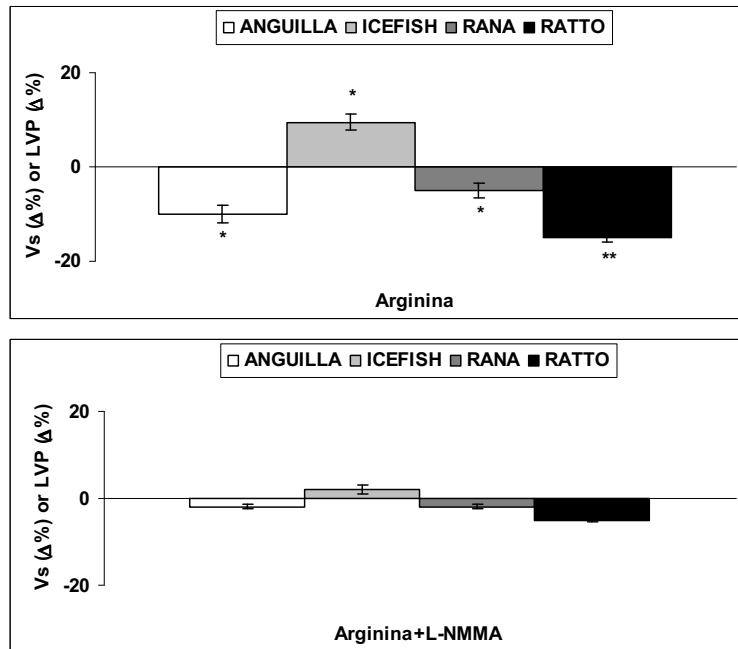
reduttasica. I cuori di anguilla, rana e ratto perfusi sono stati istantaneamente congelati in azoto liquido e al momento opportuno omogenati e sottoposti al cosiddetto “Biotin Switch Assay” (Jaffrey et al., 2001; Jaffrey & Snyder, 2001). I risultati ottenuti dimostrano che nei cuori di anguilla e ratto trattati con nitrito aumentano i livelli di nitrosilazione delle proteine di membrana e nei cuori di rana trattati con nitrito aumentano i livelli di nitrosilazione sia delle proteine di membrana sia di quelle citosoliche (*fig. 11*).

## EFFETTO INOTROPO INDOTTO DA NITRITI



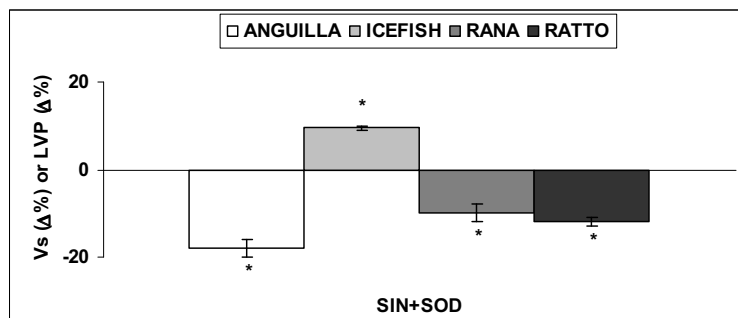
**Figura 4.** Curve dose-risposta dei nitriti valutate a 10 minuti dall'inizio del trattamento. I dati sono espressi in medie percentuali  $\pm$  l'errore standard (n=5-7). t-test: \* =  $p < 0,05$ ; \*\* =  $p < 0,01$ ; \*\*\* =  $p < 0,001$ . ANOVA: § =  $p < 0,05$ ; §§ =  $p < 0,01$ ; §§§ =  $p < 0,001$ .

## EFFETTO INOTROPO INDOTTO DA NO ENDOGENO



**Figura 5.** Effetto della L-arginina su cuore di anguilla ( $10^{-7}$  M), icefish ( $10^{-6}$  M), rana ( $10^{-7}$  M) e ratto ( $10^{-8}$  M) prima e dopo trattamento con L-NMMA ( $10^{-5}$  M). I dati sono espressi in medie percentuali  $\pm$  l'errore standard (n=4-7). t-test: \* =  $p < 0,05$ ; \*\* =  $p < 0,01$ ; \*\*\* =  $p < 0,001$ . ANOVA: § =  $p < 0,05$ ; §§ =  $p < 0,01$ ; §§§ =  $p < 0,001$ .

## EFFETTO INOTROPO INDOTTO DA NO ESOGENO



**Figura 6.** Effetto del SIN-1 (in presenza di SOD 10 IU/ml) su cuore di anguilla ( $10^{-7}$  M), icefish ( $10^{-5}$  M), rana ( $10^{-7}$  M) e ratto ( $10^{-10}$  M). I dati sono espressi in medie percentuali  $\pm$  l'errore standard (n=4-5). t-test: \* =  $p < 0,05$ ; \*\* =  $p < 0,01$ ; \*\*\* =  $p < 0,001$ . ANOVA: § =  $p < 0,05$ ; §§ =  $p < 0,01$ ; §§§ =  $p < 0,001$ .

COINVOLGIMENTO DELLE NOS

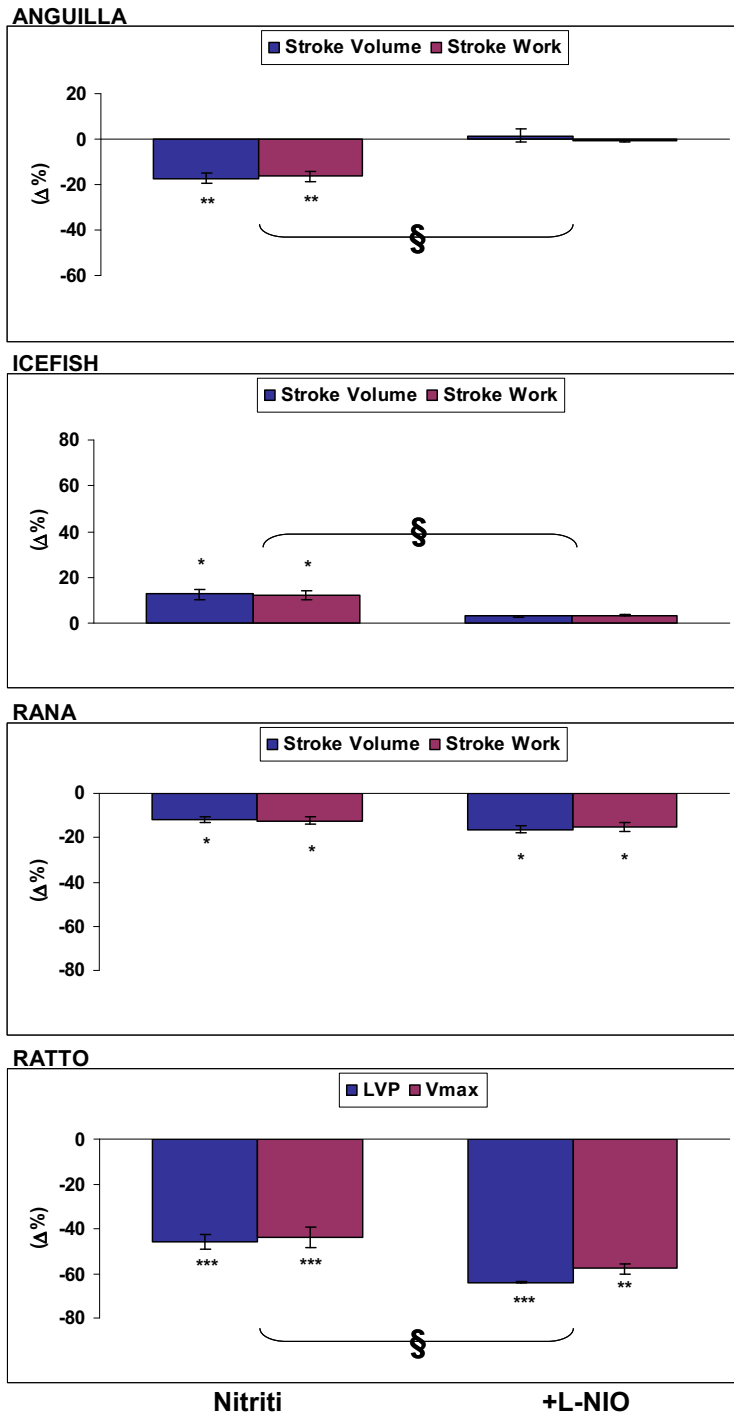
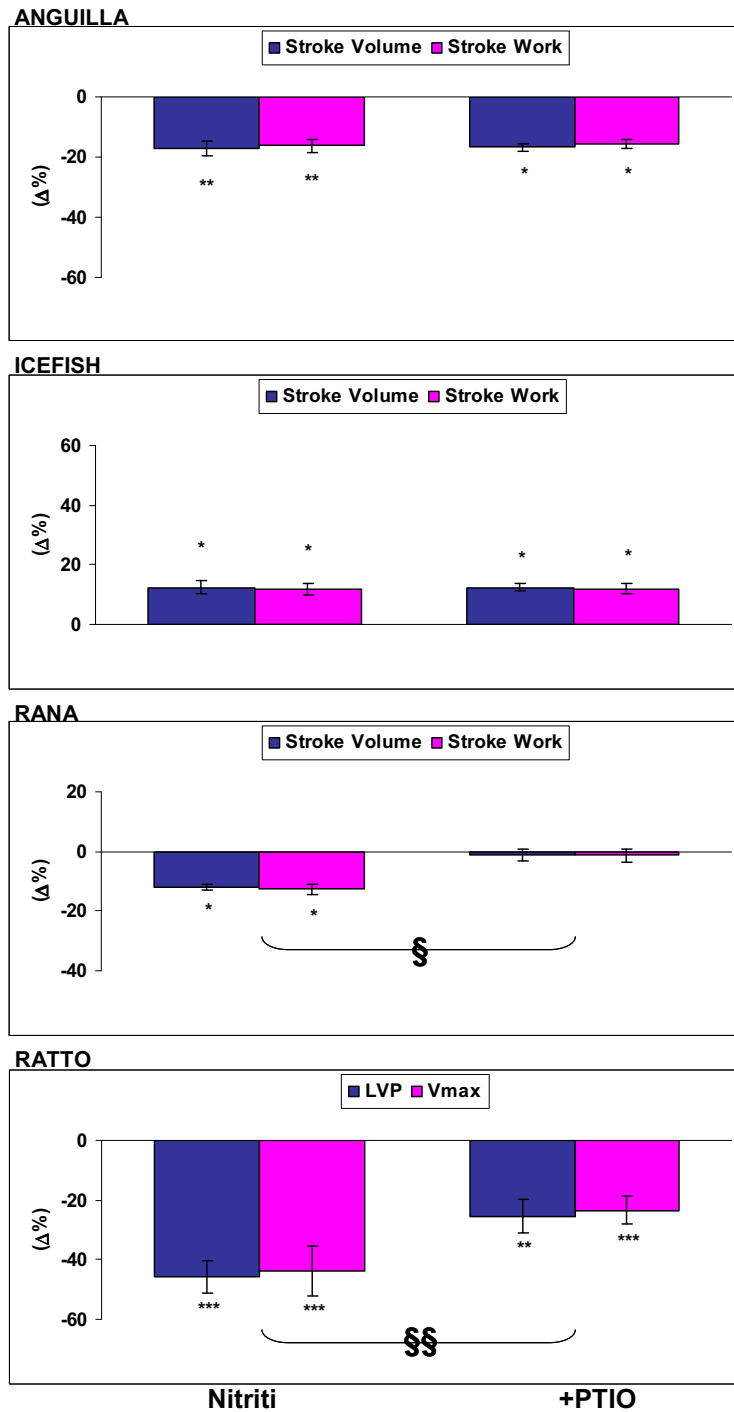


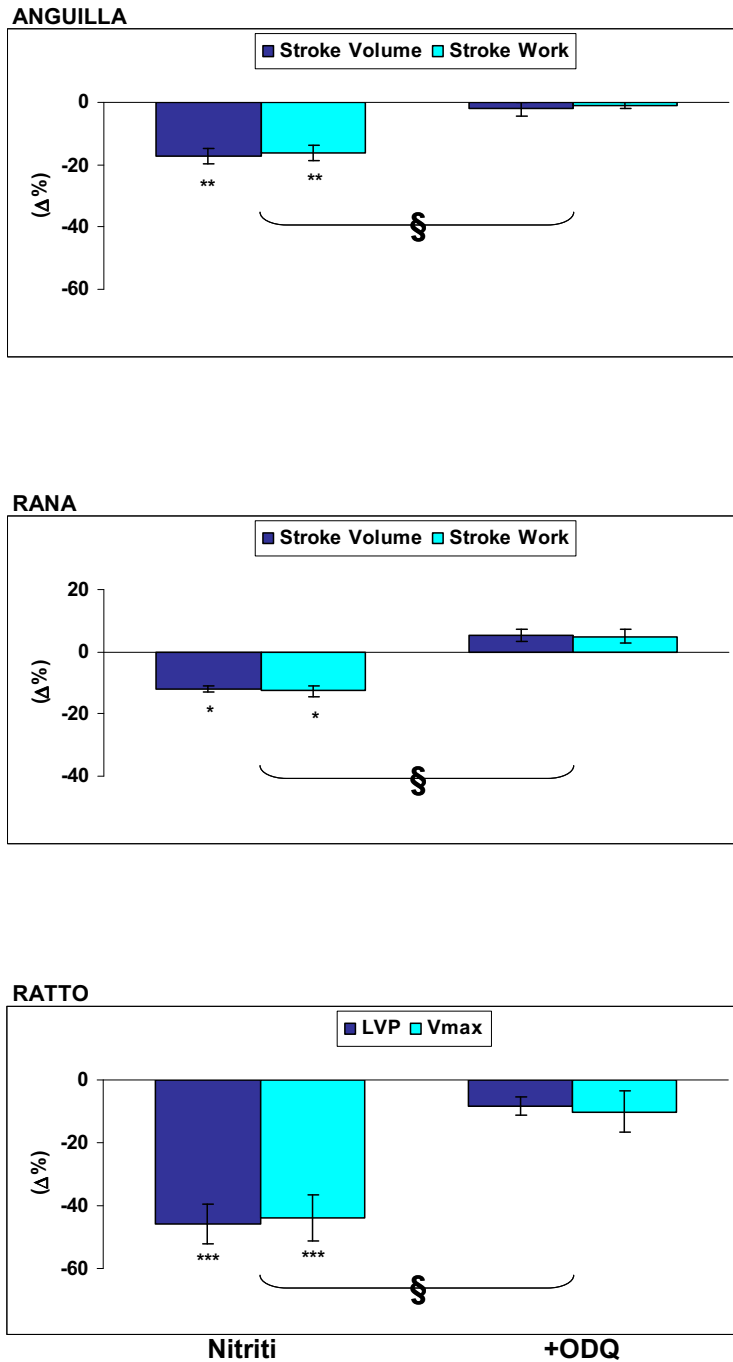
Figura 7. Effetto dei nitriti su cuore di anguilla ( $10^{-5}$  M), icefish ( $10^{-5}$  M), rana ( $10^{-5}$  M) e ratto ( $10^{-8}$  M) prima e dopo trattamento con L-NIO ( $10^{-5}$  M). I dati sono espressi in medie percentuali  $\pm$  l'errore standard (n=4-7). t-test: \* =  $p < 0,05$ ; \*\* =  $p < 0,01$ ; \*\*\* =  $p < 0,001$ . ANOVA: § =  $p < 0,05$ ; §§ =  $p < 0,01$ ; §§§ =  $p < 0,001$ .

**COINVOLGIMENTO DELLO NO**



**Figura 8.** Effetto dei nitriti su cuore di anguilla ( $10^{-5}$  M), icefish ( $10^{-5}$  M), rana ( $10^{-5}$  M) e ratto ( $10^{-8}$  M) prima e dopo trattamento con PTIO ( $10^{-7}$  M). I dati sono espressi in medie percentuali  $\pm$  l'errore standard (n=4-7). t-test: \* = p<0,05; \*\* = p<0,01; \*\*\* = p<0,001. ANOVA: § = p<0,05; §§ = p<0,01; §§§ = p<0,001.

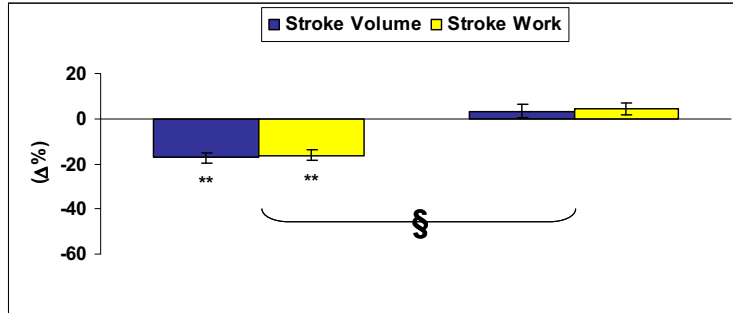
## COINVOLGIMENTO DELLA sGC



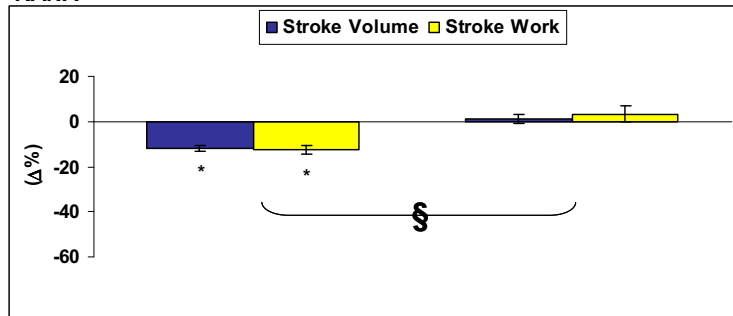
**Figura 9.** Effetto dei nitriti su cuore di anguilla ( $10^{-5}$  M), icefish ( $10^{-5}$  M), rana ( $10^{-5}$  M) e ratto ( $10^{-8}$  M) prima e dopo trattamento con ODQ ( $10^{-5}$  M). I dati sono espressi in medie percentuali  $\pm$  l'errore standard (n=4-7). t-test: \* =  $p < 0,05$ ; \*\* =  $p < 0,01$ ; \*\*\* =  $p < 0,001$ . ANOVA: § =  $p < 0,05$ ; §§ =  $p < 0,01$ ; §§§ =  $p < 0,001$ .

## COINVOLGIMENTO DELLA PKG

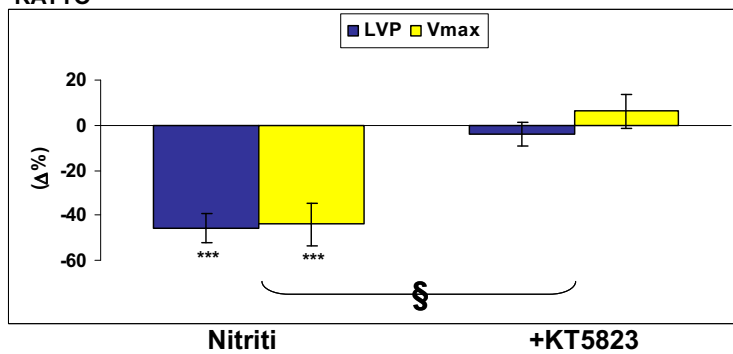
### ANGUILLA



### RANA



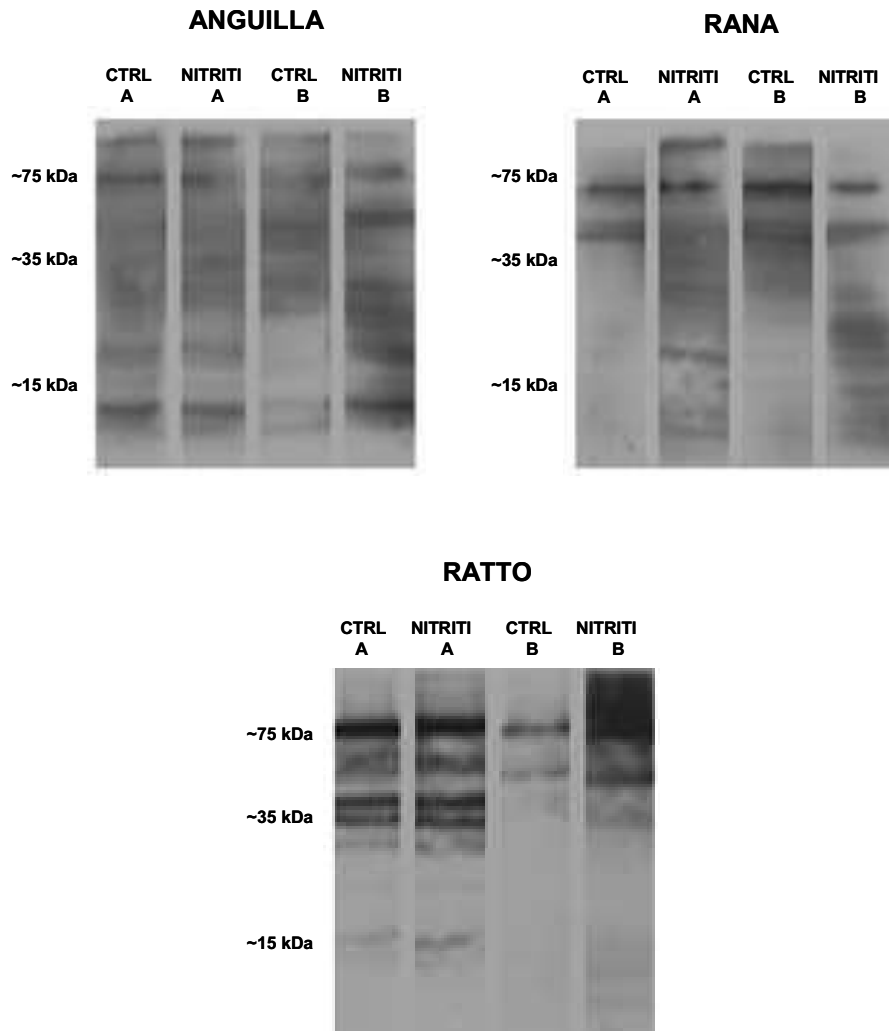
### RATTO



**Figura 10.** Effetto dei nitriti su cuore di anguilla ( $10^{-5}$  M), icefish ( $10^{-5}$  M), rana ( $10^{-5}$  M) e ratto ( $10^{-8}$  M) prima e dopo trattamento con KT5823 ( $10^{-7}$  M). I dati sono espressi in medie percentuali  $\pm$  l'errore standard (n=4-7). t-test: \* =  $p < 0,05$ ; \*\* =  $p < 0,01$ ; \*\*\* =  $p < 0,001$ . ANOVA: § =  $p < 0,05$ ; §§ =  $p < 0,01$ ; §§§ =  $p < 0,001$ .



## S-NITROSILAZIONE DELLE PROTEINE CARDIACHE



**Figura 11.** Western blot delle proteine S-nitrosilate su omogenato di cuore di anguilla, rana e ratto. CTRL: controllo; NITRITI: trattamento con nitriti. A: proteine citosoliche; B: proteine di membrana (n=3).

## ***DISCUSSIONE E CONCLUSIONI***

Questo studio condotto sul cuore isolato e perfuso di quattro vertebrati ha permesso di mettere in luce l'importante attività biologica dei nitriti in condizioni fisiologiche. Inoltre, l'analisi comparata di cuori diversi per design cardiaco ed assetto vasale si è rivelata di particolare importanza negli studi sullo NO, in quanto l'utilizzo di cuori avascolari ha consentito di valutare gli effetti diretti sul miocardio senza interferenze da parte della porzione vasale che, com'è noto, è estremamente reattiva nei confronti dello NO.

I risultati ottenuti dimostrano che i nitriti incidono potentemente sulla performance cardiaca modulando la contrattilità del cuore dei quattro modelli presi in esame. Gli effetti esercitati dai nitriti sono del tutto paragonabili a quelli esercitati dallo NO proveniente sia da fonti endogene che esogene e, inoltre, rivelano una diversa sensibilità ai nitriti che è specie-specifica.

I nitriti esercitano un effetto inotropo negativo nel cuore di anguilla, rana e ratto. Nel cuore di icefish, invece, si riscontra un effetto inotropo positivo indotto dai nitriti che rispecchia l'effetto dello NO sia endogeno che esogeno. Tali effetti possono essere attribuiti a più fattori, quali la diversa sub-compartmentalizzazione spazio-temporale delle NOS, i vari processi intracellulari conseguenti all'estrema stenotermia, l'assenza di emoglobina (Pellegrino et al., 2004; Amelio et al., 2006).

Nei pesci, l'inibizione delle NOS tramite l'inibitore L-NIO abolisce completamente l'effetto sull'inotropismo. Nella rana l'effetto indotto dai nitriti

non viene modificato dal pretrattamento con L-NIO mentre nel ratto viene potenziato. Il potenziamento dell'effetto inotropo indotto dai nitriti può dipendere da un effetto sinergico, infatti lo L-NIO esercita un effetto inotropo negativo anche se somministrato da solo. Tale potenziamento, inoltre, potrebbe essere dovuto anche al fatto che lo L-NIO da un lato blocca il sito di legame della L-arginina sulla eNOS eliminandone dunque l'attività catalitica, ma dall'altro induce l'attività reduttasica promuovendo la conversione dei nitriti in NO (Gautier et al., 2006).

Questi dati mettono in luce un diverso meccanismo che sottende all'effetto dei nitriti nelle diverse specie e suggeriscono che nei pesci i nitriti esercitano il loro effetto in presenza di una NOS funzionale, infatti, esiste la possibilità che in queste specie le NOS, in particolare la eNOS, agiscano da nitrito-reduttasi come suggerito per alcuni mammiferi (Gautier et al., 2006). Al contrario, nella rana e nel ratto l'effetto esercitato dai nitriti è completamente NOS-indipendente.

Inoltre, nel cuore di anguilla e icefish, l'effetto nitrito-dipendente che è abolito dall'inibizione delle NOS non è invece modificato da scavenger di NO, suggerendo che l'azione dei nitriti si ha in presenza di una NOS funzionale ma non dipende dalla loro riduzione a NO. Al contrario, nel cuore di rana e ratto, l'effetto esercitato dai nitriti è inibito da scavenger di NO ma non da inibitori delle NOS ed è dunque dovuto alla riduzione dei nitriti a NO attraverso un meccanismo NOS-indipendente.

L'analisi dei meccanismi di traduzione del segnale è stata effettuata sul cuore di anguilla, rana e ratto ma non sulla specie antartica per le difficoltà logistiche nel reperire il campione. I risultati ottenuti hanno dimostrato il coinvolgimento del pathway trasduzionale sGC-cGMP-PKG nel meccanismo d'azione dei nitriti. L'effetto inotropo negativo nel cuore di anguilla, rana e ratto è inibito in presenza dell'inibitore della sGC, ODQ. Tuttavia, a causa delle sue proprietà ossidanti (Feelisch et al., 1999), non si può escludere che lo ODQ inibisca l'effetto dei nitriti ossidando altre proteine cardiache, oltre alla sGC. Anche il trattamento con l'inibitore specifico della PKG, KT5823, abolisce l'effetto esercitato dai nitriti nelle tre specie prese in esame. Nel cuore di anguilla e rana, così come è ben noto nel cuore di ratto, la PKG può ridurre le correnti di calcio L-type (Imbrogno et al., 2004; Corti et al., 2002; Abi-Gerges et al., 2001) e l'affinità della troponina C per il calcio attraverso la fosforilazione della troponina I, regolando così negativamente la contrattilità (Hove-Madsen et al., 1996). Dunque sia la riduzione delle correnti di calcio sia la desensitizzazione al calcio possono contribuire all'effetto inotropo negativo esercitato dai nitriti.

Inoltre, sono stati valutati i livelli di nitrosilazione delle proteine cardiache, in quanto indice della loro eventuale azione nitrito-reduttasica. La S-nitrosilazione sta emergendo quale fondamentale meccanismo con cui lo NO media un gran numero di processi intracellulari (Hess et al., 2005). Recenti scoperte, infatti, hanno mostrato che la S-nitrosilazione regola diversi processi che coinvolgono ad esempio i recettori accoppiati a proteine

G (Whalen et al., 2007), la Hsp90 (Martinez-Ruiz et al., 2005), le cascate mitocondriali pro- e anti- apoptotiche (Choi et al., 2002). Inoltre, una irregolare S-nitrosilazione dei recettori della rianodina può contribuire allo sviluppo di aritmie e altre patologie cardiache (Gonzalez et al., 2007; Durham et al., 2008; Lehnart et al., 2008).

Dunque tempo ed energia sono stati investiti per la messa a punto di una nuova metodica, prima d'ora mai utilizzata su tessuti cardiaci in seguito a perfusione, che permette di rilevare le proteine S-nitrosilate nei nostri preparati. La messa a punto di tale metodica non è stata immediata e ha comportato diversi tentativi prima di individuare il protocollo con resa ottimale ma ha reso possibile l'applicazione del "Biotin Switch Assay" su preparati di cuore isolati e perfusi, in modo da analizzare l'effetto di trattamenti fisio-farmacologici. Ad esempio, tale metodica si è rivelata di estrema importanza nell'individuare il fosfolambano come specifico target di S-nitrosilazione nel cuore di anguilla sottoposto a stretch (Garofalo et al., 2009).

L'analisi tramite "Biotin Switch Assay" dei campioni di cuore dopo perfusione con nitriti ha rivelato un aumento dei livelli di S-nitrosilazione delle proteine. Questo fenomeno si riscontra in tutte le tre specie prese in esame. In anguilla e ratto l'aumento dei livelli di S-nitrosilazione riguarda soprattutto proteine di membrana mentre nella rana sono presenti significative variazioni sia nelle proteine di membrana che in quelle citosoliche. Studi ancora in corso sono mirati all'identificazione dei targets

finali di tale processo di S-nitrosilazione, nonché della loro localizzazione subcellulare.

In conclusione, da questo lavoro di tesi è emersa l'importante azione biologica dei nitriti in condizioni fisiologiche. Sono stati inoltre approfonditi i meccanismi d'azione di tali anioni sul cuore di differenti vertebrati che presentano un design cardiaco ben diverso sia nell'architettura ventricolare che nell'assetto vasale.

In tutti i modelli presi in esame, gli effetti esercitati dai nitriti rispecchiano quelli esercitati dallo NO proveniente sia da fonti endogene che esogene e, inoltre, presentano una specie-specifica sensibilità ai nitriti.

Nel cuore di rana e ratto, la riduzione dei nitriti a NO avviene attraverso un meccanismo NOS-indipendente; al contrario, nel cuore di anguilla e icefish, l'azione dei nitriti non è dovuta alla loro conversione a NO. In anguilla, rana e ratto, l'effetto nitrito-dipendente coinvolge il pathway sGC-cGMP-PKG e comporta aumento dei livelli di nitrosilazione delle proteine cardiache.

L'analisi comparata del complesso sistema nitrito/NO risulta di notevole interesse per chiarire l'attività biologica di questo importantissimo sistema segnale anche per fornire nuove prospettive in ambito medico e clinico.

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## PUBBLICAZIONI SCIENTIFICHE

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Nitrite modulates contractility of teleost (*Anguilla anguilla* and *Chionodraco hamatus*, i.e. the Antarctic hemoglobinless icefish) and frog (*Rana esculenta*) hearts

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### **Phospholamban S-nitrosylation modulates Starling response in fish heart**

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## Nitrite modulates contractility of teleost (*Anguilla anguilla* and *Chionodraco hamatus*, i.e. the Antarctic hemoglobinless icefish) and frog (*Rana esculenta*) hearts

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### ABSTRACT

Being the largest form of intravascular and tissue storage of nitric oxide (NO) and a signalling molecule itself, the nitrite anion ( $\text{NO}_2^-$ ) has emerged as a key player in many biological processes. Since the heart is under an important NO-mediated autocrine–paracrine control, in mammals the cardiac effects of nitrite are under intensive investigation. In contrast, nothing is known in non-mammalian vertebrates. We evaluated nitrite influence on cardiac performance in the perfused beating heart of three different cold-blooded vertebrates, i.e. two teleost fishes, the temperate red-blooded *Anguilla anguilla*, the Antarctic stenotherm, hemoglobinless *Chionodraco hamatus* (icefish), and the frog *Rana esculenta*. We showed that, under basal conditions, in all animals nitrite influences cardiac mechanical performance, inducing negative inotropism in eel and frog, while being a positive inotrope in *C. hamatus*. In all species, these responses parallel the inotropic effects of authentic NO. We also demonstrated that the nitrite-dependent inotropic effects are i) dependent from NO synthase (NOS) activity in fish; ii) sensitive to NO scavenging in frog; iii) cGMP/PKG-dependent in both eel and frog. Results suggest that nitrite is an integral physiological source of NO and acts as a signalling molecule in lower vertebrate hearts, exerting relevant inotropic actions through different species-specific mechanisms.

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### 1. Introduction

Over recent years, the biology of inorganic nitrogen oxides has been the subject of many investigations. Although knowledge on their biological effects of nitrite has a long and rich history, the emerging role of nitrite as a nitric oxide (NO) reservoir in mammals, including man, opened the door to novel implications both at physiological and pathological levels [1,2].

Nitrite anion ( $\text{NO}_2^-$ ) is relatively abundant in blood and tissues and its plasma levels are conserved across various mammalian species, including man, in the range of 150–600 nmol  $\text{l}^{-1}$  and may increase slightly in response to inflammation or infection [3,4]. It was estimated that as much as 70% of plasma nitrite originates from nitric oxide synthases (NOSs), mainly in the endothelium by endothelial NOS (eNOS). In fact, nitrite has been reported as an index of NOSs activity [1]. In addition to endogenous generation, exogenous sources, principally environmental pollutants and intake of vegetables, contribute to the build-up of this NO reserve [5].

A growing body of evidence suggests that nitrite anion, rather than being an inert waste product of NO metabolism, may be a key player in

biological processes, representing, on one hand, the largest form of intravascular and tissue storage pool of NO and, on the other hand, a signalling molecule itself [6].

Mechanisms for the *in vivo* conversion of nitrite to NO may involve either enzymatic reduction or non-enzymatic reduction [see 2 for references]. Some proteins show nitrite reductase capacity, i.e. glutathione-S-transferases, xanthine oxidoreductase, cytochrome P-450 enzymes, deoxy-hemoglobin and deoxy-myoglobin, as well as eNOS [7,8]. Each mechanism would occur preferentially during pathological hypoxia and acidosis [9] present in disease states, such as ischemia [10]. In fact, during ischemia this alternative NO production may exert a protective influence because L-arginine-NOS-derived NO generation depends on oxygen, which is rapidly depleted under ischemic conditions. Accordingly, recent evidence is consistent with a powerful protective influence of nitrite in animal models of ischemia–reperfusion injury [10,11]. It was suggested that nitrite acts via formation of NO, but there is also a possibility that nitrite can modulate physiological processes via other pathways, for example through direct S-nitrosylation of thiol-containing proteins.

Recently, some authors have reported that nitrite has a distinct and important signalling role under normal physiological conditions, being capable of modulating many important signalling pathways, including soluble guanylyl cyclase (sGC) stimulation, cytochrome P-450 activity and the expression of two archetypical proteins, heat shock protein 70 (Hsp 70) and heme oxygenase-1 (HO-1) [12]. Nitrite

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is a quantitative relevant component of the environment of cold-blooded vertebrates, particularly fish and amphibians, and therefore we may expect that in these animal groups the anion-elicited effects may be, at least, as important as in mammals. Furthermore, many fish and amphibians are chronically exposed to the challenge of environmental hypoxia and acidosis. For the first time in fish, Jensen [13] tested the hypothesis that  $\text{NO}_2^-$  accumulation across the gills of zebrafish causes a relevant production of NO from nitrite. He used deconvolution spectral methodology and blood level of nitrosylhemoglobin (HbNO), as a biomarker of intra-organismal NO generation, and showed very high HbNO levels in fish exposed for variable time periods to different doses of nitrite (dissolved as  $\text{NaNO}_2$  in the environmental water). This finding is consistent with deoxyHb-mediated reduction of nitrite as a major NO-producing mechanism which appears physiologically important in the *in vivo* arterial-venous circulation when Hb circulates between full and intermediate oxygen saturation. The aim of this research was to explore the biological activity of nitrite as a putative signalling molecule under basal conditions in isolated and perfused working hearts (teleost fishes and frog), selected as appropriate case study models of lower vertebrate cardiac physiologies.

We used the temperate eurytherm European eel (*Anguilla anguilla*) and the cold-adapted Antarctic *Chionodraco hamatus* (icefish) and the frog *Rana esculenta*. Both eel and frog experience considerable fluctuations of environmental oxygen, the former being a champion of prolonged hypoxia and acidosis tolerance. In contrast, the icefish represents a unique case of extreme stenothermia; in fact, it is an endemic inhabitant of the stably icy and richly oxygenated Antarctic waters. Conceivably, these environmental conditions have allowed the animal to survive despite its paradoxical loss of hemoglobin and red blood cells, normally indispensable for adult vertebrate life [see 14,15 for references]. Since Hb is a key protein in NO homeostasis, functioning both as NO scavenger and generator from nitrite, the hemoglobinless icefish provides exclusive opportunities to investigate NO/nitrite signalling in a naturally occurring genetic knockout for Hb. This comparative analysis may be of relevance to understand the biological activity of nitrite in modulating the “normal” function of the vertebrate heart, verifying, at the same time, to which extent aspects of cardiovascular regulation and even classification of NO and nitrite-dependent mechanisms can be applied to representatives of more than one group (mammals) of vertebrates.

## 2. Materials and methods

### 2.1. Chemicals

Biotin-HPDP was purchased from Pierce; ECL was purchased from Amersham; the other chemicals/drugs were purchased from Sigma unless otherwise indicated and prepared immediately before each experiment. ODQ was prepared in ethanol, while the other solutions were prepared in double-distilled water. KT5823 was used in a darkened perfusion apparatus to prevent degradation.

### 2.2. Animals

European eels (*A. anguilla* L.) and frogs (*R. esculenta*) of both sexes, weighing 90–120 g and 15–25 g respectively, were provided by a local hatchery and kept at room temperature (18–20 °C) without feeding for 5–7 days.

Male and female icefish (*C. hamatus*), weighing 320–370 g, were caught by nets in the Terranova Bay, Ross Sea, Antarctica during January 2005. The animals were maintained unfed for at least 5 days in aerated, running seawater at temperatures between 0 and 2 °C. The experiments were done in the Italian Antarctic Base laboratories, Terranova Bay (74°42'S, 164°06'E), during the XX Italian Antarctic Expedition (December 2004–February 2005).

All animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

### 2.3. Isolated and perfused working heart preparations

Both fish (eel and icefish) were anaesthetized in benzocaine ( $0.2 \text{ g l}^{-1}$ ) for 15 min and were opened ventrally. The ventral aorta was cannulated and the heart removed without the pericardium and placed in an ice-chilled dish filled with saline for the atrium cannulation procedure. A polyethylene cannula was secured in the atrium at the junction with the *sinus venosus*. Isolation time was 5 min.

Frogs were pited and ventrally opened; the pericardium was removed and the heart cannulated *in situ* with polyethylene cannula inserted into the *sinus venosus* for the inflow and into the left aortic trunk for outflow collection. Right aortic trunk, right and left *praecava* veins and pulmonary vein were tied off by ligature. Isolation time was 15–20 min.

The cannulated heart was transferred to a perfusion chamber filled with saline and connected with a perfusion apparatus as previously described [eel, 16; icefish, 17; frog, 18]. Perfusion was immediately started; the heart received saline from an input reservoir pumped against an afterload pressure given by the height of an output reservoir.

The hearts were perfused with Ringer's solution at constant input pressures and were able to produce physiological values of work and power [eel, 16; icefish, 17; frog, 18]. The experiments on eel and frog were performed at room temperature (18–20 °C), the experiments on icefish were performed in a cold thermostatted cabinet (LKB 2021 Maxicoldlab, Malbo, Sweden), which allowed the heart and perfusion system to be maintained at near zero temperatures. The control conditions were: *A. anguilla*, mean output pressure 3.00 kPa with cardiac output set to  $10 \text{ ml min}^{-1} \text{ kg}^{-1}$  body mass; *C. hamatus*, mean output pressure 1.4 kPa with cardiac output set to  $50 \text{ ml min}^{-1} \text{ kg}^{-1}$  body mass; *R. esculenta*, mean output pressure 3.92 kPa with cardiac output set to  $110 \text{ ml min}^{-1} \text{ kg}^{-1}$  body mass. Cardiac output was set by appropriately adjusting filling pressure.

Heart rate (HR) was calculated from pressure recording curves. Cardiac output (CO) was collected over 1 min and weighed; values were corrected for fluid density and expressed as volume measurements. Stroke volume ( $V_s$ ;  $\text{ml kg}^{-1}$ ;  $\text{CO}/\text{HR}$ ) was used as a measure of ventricular performance; changes in  $V_s$  were considered to be inotropic effects. CO and  $V_s$  were normalized per kilogram of wet body mass. Ventricular stroke work [ $W_s$ ;  $\text{mJ g}^{-1}$ ;  $(\text{afterload-preload}) \times V_s/\text{ventricle mass}$ ] served as an index of systolic functionality.  $W_s$  was corrected for the ventricle weights (eel,  $0.065 \pm 0.003$ ; icefish,  $1.12 \pm 0.23$ ; frog,  $0.048 \pm 0.002$ ) measured at the end of each experiment after the removal of excess water.

### 2.4. Experimental protocol

In all experiments, hearts were allowed to equilibrate to conditions which simulate an *in vivo* resting state for up to 15–20 min. Hearts that did not stabilize within 20 min of perfusion were discarded. Cardiac variables were measured simultaneously during experiments. All experiments were carried out on the basis of endurance profile for each species (eel, 120 min, [16]; icefish, 120 min, [17]; frog, 80 min, [18]). To avoid the influence of chronotropism on inotropism, all the hearts were electrically paced at spontaneous rhythm. However, control experiments on unpaced heart (data not shown) revealed that nitrite did not modify HR.

The response of the hearts to pharmacological agents ( $\text{L-arginine}$ : eel  $10^{-7} \text{ mol l}^{-1}$ , icefish  $10^{-6} \text{ mol l}^{-1}$  [17], frog  $10^{-7} \text{ mol l}^{-1}$ ;  $\text{L-NMMA}$ :  $10^{-5} \text{ mol l}^{-1}$ ;  $\text{SIN-1}$ : eel  $10^{-7} \text{ mol l}^{-1}$ , icefish  $10^{-5} \text{ mol l}^{-1}$  [17], frog  $10^{-7} \text{ mol l}^{-1}$ ;  $\text{SOD}$ : 10 IU/ml;  $\text{L-NIO}$ :  $10^{-5} \text{ mol l}^{-1}$ ;  $\text{PTIO}$ :



**Table 1**  
Baseline cardiac parameters in working hearts.

Species	Heart rate (beats/min)	Filling pressure (kPa)	Output pressure (kPa)	Cardiac output (ml min <sup>-1</sup> kg <sup>-1</sup> )	Stroke volume (ml min <sup>-1</sup> kg <sup>-1</sup> )	Stroke work (mJ g <sup>-1</sup> )
Eel	51.4 ± 12.2	0.07 ± 0.008	2.97 ± 0.034	10.9 ± 1.56	0.21 ± 0.09	0.105 ± 0.03
Icefish	31.2 ± 0.79	0.117 ± 0.011	1.385 ± 0.085	50.5 ± 3.62	1.861 ± 0.113	2.241 ± 0.258
Frog	55.4 ± 2.8	0.5 ± 0.05	3.76 ± 0.1	107.4 ± 3.62	1.96 ± 0.39	4.15 ± 0.32

10<sup>-7</sup> mol l<sup>-1</sup>; ODQ: 10<sup>-5</sup> mol l<sup>-1</sup>; KT5823: 10<sup>-7</sup> mol l<sup>-1</sup>) was obtained by perfusing each cardiac preparations with the buffer containing only one chemical at the desired concentration in the presence or absence of increasing concentrations of sodium nitrite.

### 2.5. Statistics

Data were expressed as the mean ± SEM. Since each heart represents its own control, the statistical significance of differences within-group was assessed using the paired Student's *t*-test (*P* < 0.05). Comparison between groups was made using a one-way analysis of variance (ANOVA) followed by Duncan's test. Differences were considered to be statistically significant for *P* < 0.05.

### 2.6. Homogenization protocol

Hearts were homogenized on ice in 20 mmol l<sup>-1</sup> Tris pH 7.5, 150 mmol l<sup>-1</sup> NaCl, 1% Igepal CA 630, 0.5% Sodium Deoxycholate, 1 mmol l<sup>-1</sup> EDTA, 0.1% SDS, 200 mmol l<sup>-1</sup> Sodium Orthovanadate, and Protease Inhibitor Cocktail, using a polytron tissue grinder. The homogenate was centrifuged at 4 °C for 40 min at 13,000 *g*. The supernatant containing cytosolic proteins was collected and proteins were quantified with Bradford reagent; the pellet containing membrane proteins was resuspended in homogenization buffer and proteins were quantified with Bradford reagent.

### 2.7. Biotin switch assay and Western blot

The biotin switch assay was performed essentially as previously described [19]. Extracts were adjusted to 0.5 mg/ml of protein and equal amounts were blocked with 4 volumes of blocking buffer (225 mmol l<sup>-1</sup> Hepes, pH 7.7, 0.9 mmol l<sup>-1</sup> EDTA, 0.09 mmol l<sup>-1</sup> neocuproine, 2.5% SDS, and 20 mmol l<sup>-1</sup> MMTS) at 50 °C for 20 min with agitation. After blocking, extracts were precipitated with 2 volumes of cold (-20 °C) acetone, chilled at -20 °C for 10 min, centrifuged at 2000 *g*, 4 °C for 5 min, washed with acetone, dried out at room temperature and resuspended in 0.1 ml HENS buffer (250 mmol l<sup>-1</sup> Hepes, pH 7.7, 1 mmol l<sup>-1</sup> EDTA, 0.1 mmol l<sup>-1</sup> neocuproine, and 1% SDS) x mg of protein. Until this step, all operations were carried out in the dark. A 1/3 vol of biotin-HPDP 4 mmol l<sup>-1</sup> in DMF and ascorbate 1 mmol l<sup>-1</sup> were added and incubated for 1 h at room temperature. Proteins were acetone-precipitated again and resuspended in the same volume of HENS buffer.

To detect biotinylated proteins by Western blot, samples from the biotin switch assay were separated on 15% SDS-PAGE gels, transferred to PVDF membranes, blocked with non fat dried milk, and incubated with streptavidin-peroxidase diluted 1/5000 for 1 h.

Blots were developed by enhanced chemiluminescence (ECL) and were placed in a film cassette with photograph film. Films were exposed for 30 s, developed and fixed.

## 3. Results

### 3.1. Isolated and perfused working heart preparations

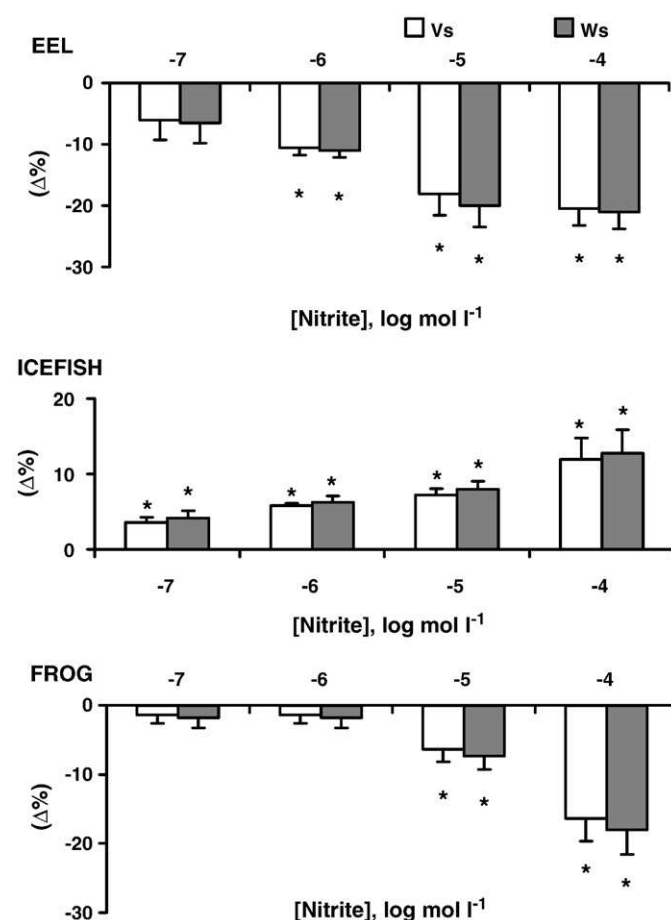
Under basal conditions, the hemodynamic characteristics of the isolated and perfused working heart preparations from eel, icefish,

and frog mimic the physiological values of the *in vivo* animal. Baseline hemodynamic parameters of all species (heart rate, filling pressure, output pressure, cardiac output, stroke volume, and stroke work) were measured after 15–20 min of equilibration (Table 1). The icefish cardiac output shown in Table 1 is an *in vitro* value obtained by us using the preparation of isolated and perfused working heart. This value is very close to the cardiac output calculated *in vivo* (61 ml min<sup>-1</sup> kg<sup>-1</sup> [20]).

### 3.2. Nitrite modulation of inotropism

To assess the effects of nitrite on cardiac function, the isolated perfused cardiac preparations from eel, icefish, and frog were exposed to nitrite and parameters of cardiac function were measured. In control experiments repeated exposures of each heart to the same concentration of nitrite did not result in pharmacological tolerance (data not shown), therefore cumulative concentration-response curves were generated.

In both eel and frog, nitrite induced a concentration-dependent negative inotropic effect, shown by a decrease in stroke volume and



**Fig. 1.** Cumulative dose-response curves of nitrite on stroke volume (Vs) and stroke work (Ws) in isolated and perfused hearts of eel, icefish and frog (*n* = 5). Percentage changes were evaluated as means ± S.E.M. Asterisks indicate values significantly different from the control value: \**P* < 0.05.

stroke work, significant from  $10^{-6}$  mol  $l^{-1}$  in the eel and from  $10^{-5}$  mol  $l^{-1}$  in the frog. In contrast, in the hemoglobineless icefish, increasing concentrations of nitrite positively modulated inotropism, as indicated by an increase in stroke volume and stroke work, significant from  $10^{-7}$  mol  $l^{-1}$  (Fig. 1).

### 3.3. Inotropic effects induced by authentic nitric oxide

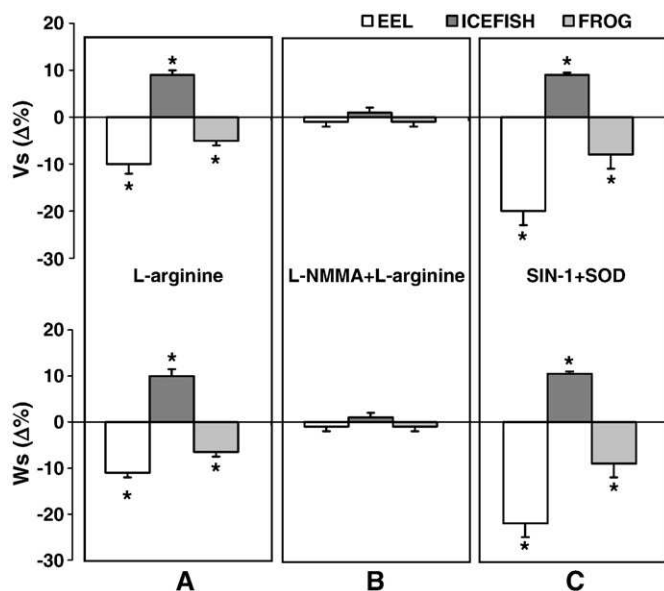
To determine the effects of endogenously generated NO on cardiac function, we treated the isolated heart preparations with the physiological substrate for NO synthase (NOS), L-arginine and a pure NOS-independent NO donor, SIN-1. We tested several doses of L-arginine and SIN-1 and we have chosen the lowest concentration which is able to induce significant effects.

As shown in Fig. 2A, similar to the effects observed with nitrite, stroke volume and stroke work were significantly decreased by L-arginine in eel and frog, while they were increased in the icefish. In all species, the L-arginine-dependent effects were inhibited by the NOS inhibitor L-NMMA ( $10^{-5}$  mol  $l^{-1}$ ), confirming that the inotropic effects of L-arginine were indeed NOS dependent (Fig. 2B).

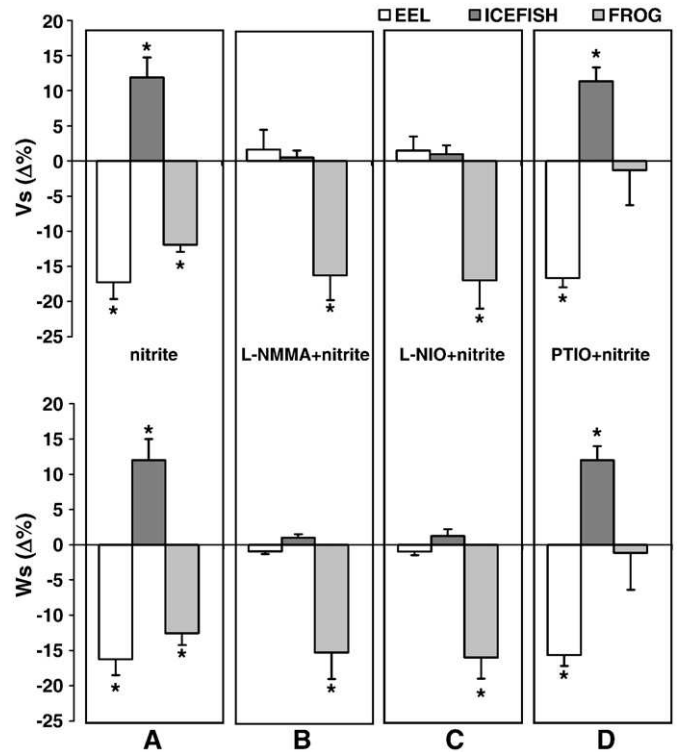
Administration of SIN-1 in the presence of superoxide dismutase, induced a negative inotropism in eel and frog and, in contrast again, a positive inotropism in icefish (Fig. 2C).

### 3.4. NOS-NO involvement in the nitrite-dependent inotropy

Since NO and nitrite similarly affect the heart, to clarify whether the inotropic effect of nitrite involves NOS-produced NO, we exposed the isolated hearts of all species to nitrite ( $10^{-5}$  mol  $l^{-1}$ ) alone (Fig. 3A) and in the presence of the NOS inhibitors L-NMMA ( $10^{-5}$  mol  $l^{-1}$ ; Fig. 3B) or L-NIO ( $10^{-5}$  mol  $l^{-1}$ ; Fig. 3C). These treatments abolished nitrite-dependent negative inotropism in eel and positive inotropism in icefish. In contrast, in the frog heart NOS inhibition did not modify the nitrite-induced negative inotropism (Figs. 3B, C). To test whether nitrite reduction to NO is involved in the nitrite-dependent inotropy, we exposed the isolated hearts of all



**Fig. 2.** Effect of L-arginine on stroke volume (Vs) and stroke work (Ws) in isolated and perfused hearts of eel ( $10^{-7}$  mol  $l^{-1}$ ), icefish ( $10^{-6}$  mol  $l^{-1}$ ) and frog ( $10^{-7}$  mol  $l^{-1}$ ) before (A) and after (B) treatment with L-NMMA ( $10^{-5}$  mol  $l^{-1}$ ) ( $n=4-7$ ). (C) Effect of SIN-1 (in presence of SOD 10 IU/ml) on stroke volume (Vs) and stroke work (Ws) in eel ( $10^{-7}$  mol  $l^{-1}$ ), icefish ( $10^{-5}$  mol  $l^{-1}$ ) and frog ( $10^{-7}$  mol  $l^{-1}$ ) ( $n=4-5$ ). Percentage changes were evaluated as means  $\pm$  S.E.M. Asterisks indicate values significantly different from the control value:  $*P<0.05$ .



**Fig. 3.** Effect of nitrite ( $10^{-5}$  mol  $l^{-1}$ ) on stroke volume (Vs) and stroke work (Ws) in isolated and perfused hearts of eel, icefish and frog before (A) and after treatment with L-NMMA ( $10^{-5}$  mol  $l^{-1}$ ) (B) or L-NIO ( $10^{-5}$  mol  $l^{-1}$ ) (C) or PTIO ( $10^{-7}$  mol  $l^{-1}$ ) (D) ( $n=4-6$ ). Percentage changes were evaluated as means  $\pm$  S.E.M. Asterisks indicate values significantly different from the control value:  $*P<0.05$ .

species to nitrite ( $10^{-5}$  mol  $l^{-1}$ ) alone (Fig. 3A) and in the presence of a NO scavenger PTIO ( $10^{-7}$  mol  $l^{-1}$ ; Fig. 3D). PTIO did not modify the nitrite-induced inotropism in both eel and icefish, while it abolished the negative inotropic effects exerted by nitrite in frog (Fig. 3D).

### 3.5. cGMP-PKG involvement in the nitrite-dependent inotropy

In the mammalian heart, activation of the NO-sGC pathway can mediate negative inotropy. To verify the involvement of cGMP-PKG cascade, cardiac preparations of eel and frog were perfused, in presence of nitrite, either with ODQ ( $10^{-5}$  mol  $l^{-1}$ ), an oxidant and a soluble guanylate cyclase inhibitor, or with a protein kinase G blocker KT5823 ( $10^{-7}$  mol  $l^{-1}$ ). (Obvious logistic difficulties in Antarctica prevented in icefish parallel experiments described in this and in the following section). As shown in Fig. 4B, in both species ODQ and KT5823 treatments abolished the negative inotropic effect of nitrite, supporting the involvement of the NO-sGC-PKG pathway in the nitrite actions (Fig. 4C).

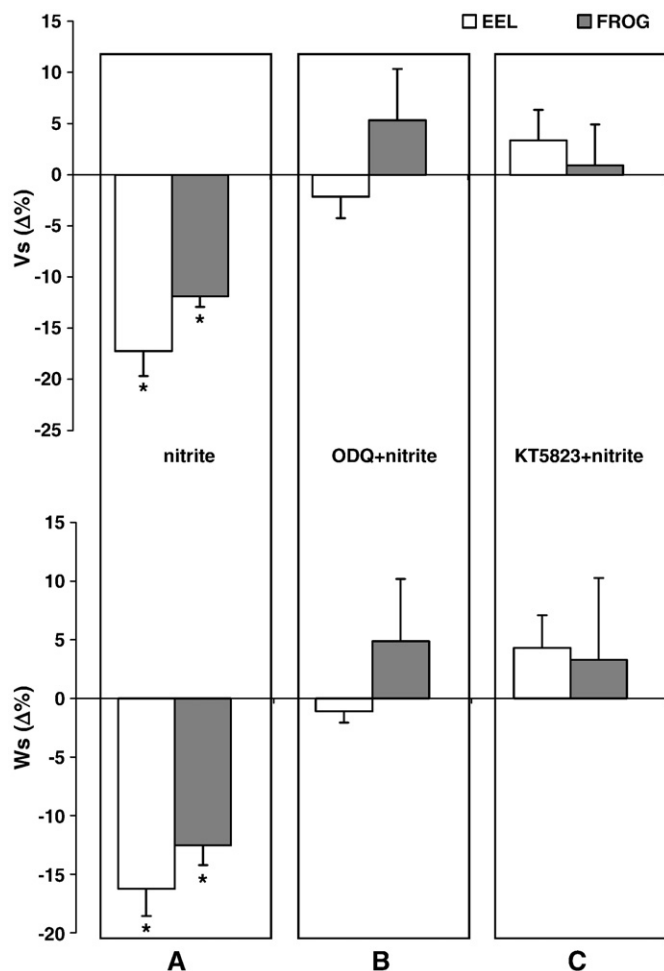
### 3.6. Analysis of S-nitrosylated proteins

In the eel cardiac tissues, the analysis of S-nitrosylated proteins revealed no differences in the cytosolic fractions, while in the membrane fractions a major expression of both low and high molecular weight S-nitrosylated proteins were present (Fig. 5).

In the frog cardiac tissues, the analysis of S-nitrosylated proteins revealed differences in both the cytosolic and membrane fractions, in particularly at low molecular weight (Fig. 6).

## 4. Discussion

Using *in vitro* isolated and perfused working heart preparations of teleost fish, i.e. the eel *A. anguilla* and the hemoglobinless icefish *C.*



**Fig. 4.** Effect of nitrite ( $10^{-5}$  mol  $l^{-1}$ ) on stroke volume ( $V_s$ ) and stroke work ( $W_s$ ) in isolated and perfused hearts of eel and frog before (A) and after treatment with ODQ ( $10^{-5}$  mol  $l^{-1}$ ) (B) or KT5823 ( $10^{-7}$  mol  $l^{-1}$ ) (C) ( $n=3-5$ ). Percentage changes were evaluated as means  $\pm$  S.E.M. Asterisks indicate values significantly different from the control value: \* $P<0.05$ .

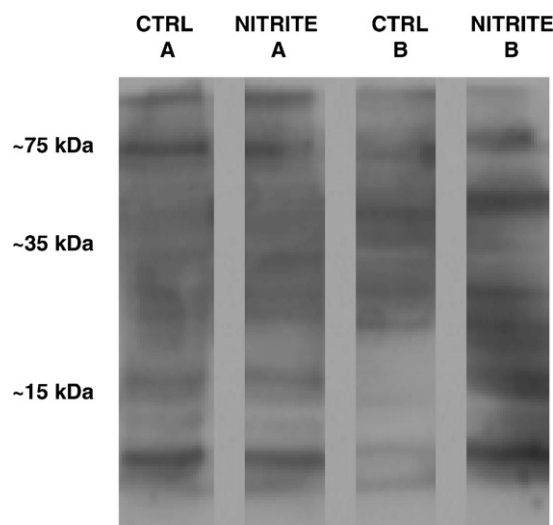
*hamatus*, as well as the frog *R. esculenta*, characterized by diverse life histories and ecophysiological traits, we provided heart paradigms well suited for highlighting the signalling properties of nitrite in a comparative vertebrate context. In particular, the distinct morpho-functional traits (see Table 2 for references) of these heart designs (fully trabeculated myoarchitecture supplied by avascular intertrabecular system of *lacunae*) allow assessment of nitrite-elicited contractile myocardial effects free from concomitant vascular (coronary) responses. Like in the highly vascularized mammalian heart [i.e. rat, 21], we found that nitrite exerts a remarkable contractile influence on the heart of the three species, regardless of their difference in vascular organization, i.e. avascular (frog, icefish) or poorly (eel) vascularized types of ventricles. We showed that nitrite negatively affects cardiac mechanical performance by decreasing stroke volume in eel and frog, while increasing it in the icefish. Of note, these nitrite-induced opposite myocardial effects parallel those induced by the direct cardiac exposure to NO [eel, 16; icefish, 17; frog, 18], providing a striking example of the role of nitrite reduction to NO in the heart. It is conceivable that the positive inotropic response observed in the icefish involves the hemoglobinless condition. The already documented [17] species-specific differences in the cardiac response to NO between the icefish and the eel, cannot be attributed to the experimental hierarchic level of investigation or to macroscopic differences in cardiac structure. The possibility exists that the very

expanded mitochondrial compartment of the icefish myocytes [17], plays a role. In zebrafish, exposure to water nitrite ( $0.6-2$  mmol  $l^{-1}$ ) was shown to induce an increase of Hb-NO levels which indicates an augmented NO production [13]. This perturbation in NO homeostasis was suggested to be responsible for important cardiovascular effects, including vasodilation and decreased blood pressure, quickly counteracted by increased cardiac pumping [22].

Interestingly, although the icefish lacks Hb, its cardiac tissue contains myoglobin which may contribute to NO generation by functioning as nitrite reductase [23]. It is possible that the nitrite-induced positive inotropism observed in the icefish is due either to a distinct spatio-temporal sub-compartmentalization of NOS isoforms, or to intracellular effectors related to extreme stenothermia, or to the absence of Hb [17,24].

Interestingly, in contrast to the cardiac responses in eel and frog, the icefish heart shows a higher sensitivity to nitrite, the first significant increase in stroke volume being obtained at  $0.1$   $\mu$ mol  $l^{-1}$ . In aquatic organisms, nitrite sensitivity shows large species variations [25]. As shown in many types of myocardial preparations, the shift from nanomolar to micromolar concentrations of intramyocardial NO and cGMP has been correlated to either positive or negative inotropic responses, respectively [see 26 for references]. The reason for the increased nitrite sensitivity of the icefish heart is unclear although several factors, to be mechanistically investigated in the future, may play a role, including the extreme cold adaptation and the stable oxygen-rich Antarctic waters, as well as the reactive oxygen species generating/scavenging abilities associated to the Hb-less condition.

Noteworthy, in the frog heart, the nitrite-dependent negative inotropism is inhibited by NO scavengers but not by NOS inhibitors, a result consistent with the reduction of nitrite to NO through a NOS-independent mechanism, as reported in the rat [21]. Contrarily, in both eel and icefish, NOS inhibition abolishes the nitrite-induced inotropic effects, which, however, are not affected by the NO scavenger PTIO, thus suggesting that nitrite action is not via its reduction to NO. Indeed, a mammalian-oriented study has suggested that nitrite may also mediate effects independent of NO generation [12]. The mechanistic explanation of the different pattern between fish and frog remains to be clarified. Since in fish a functional NOS appears essential for the inotropic effects exerted by nitrite, the possibility exists that in these piscine species the NOS isoforms, particularly eNOS, may act as nitrite-reductase, as suggested in some mammals [7].



**Fig. 5.** Western blot analysis of S-nitrosylated proteins in eel heart homogenates. Cytosolic (A) and membrane protein fractions (B). CTRL: control; NITRITE: nitrite treated-hearts.

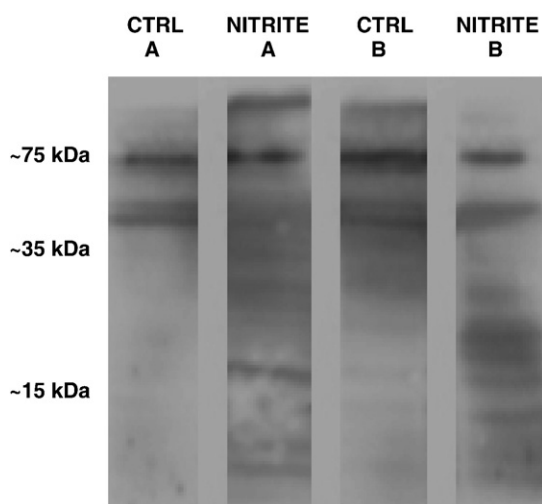


It was previously demonstrated that the hearts of eel, icefish and frog have a functioning NO axis which modulates mechanical performance via a NO-dependent increase in cGMP [16–18]. Also in the mammalian heart, several studies have demonstrated a NO-cGMP-dependent modulation of inotropism [26–29]. In all cases, the NOS system has been regarded as the exclusive physiological source of NO. However, here we show that in the heart of eel, icefish and frog, physiological levels of nitrite may also be integral sources of NO, thus strongly supporting the generalized picture emerged in mammalian-oriented studies. Noteworthy, as described in mammals, nitrite conversion to NO may occur along oxygen and pH gradients [see 30 for references], a condition which can be of importance in fish and amphibians, often exposed to chronic environmental hypoxia [31,32].

In eel and frog, the inhibition of the nitrite-dependent decrease in stroke volume in presence of ODQ suggests the involvement of the soluble guanylate cyclase-cGMP axis. Due to the oxidant properties of ODQ [33], we cannot exclude that this inhibitor interferes with the reductase ability of tissue heme proteins, thus limiting the conversion of nitrite to NO. An important intramyocardial target of cGMP is PKG [34,35]. The finding that KT5823, an inhibitor structurally unrelated to cGMP, abolished the nitrite-induced inotropism in eel and frog is consistent with a cGMP-PKG-dependent mechanism. Interestingly, also in the rat heart nitrite effects were abolished by PKG inhibitor [36, this issue]. In both eel and frog hearts [34], PKG, as shown in the mammalian myocardium, may reduce L-type  $\text{Ca}^{2+}$  currents [37] and through troponin I phosphorylation reduces troponin C affinity for  $\text{Ca}^{2+}$ , thus negatively affecting contractility [38]. We suggest that a reduction of L-type  $\text{Ca}^{2+}$  current and a PKG-mediated myofilament desensitization to  $\text{Ca}^{2+}$  may contribute to the nitrite-induced negative inotropism in eel and frog.

Using the biotin switch method for the specific tagging of S-nitrosylated proteins [19], we evaluated the level of nitrosylated proteins in the hearts of eel and frog treated with nitrite. Protein S-nitrosylation, the covalent addition of a nitroso group to a cysteine thiol side chain, has recently emerged as a major mechanism by which NO mediates a large number of intracellular processes [39]. Our results showed that nitrite-derived NO induces an increase of protein S-nitrosylation in both membrane and cytosolic fractions of frog and in membrane fraction of eel. A study in our laboratory is in progress to identify the final molecular targets of this nitrosylation process and their subcellular localization.

Recent findings have shown that S-nitrosylation regulates diverse pathways, including G-protein-coupled receptor signalling [40,41],



**Fig. 6.** Western blot analysis of S-nitrosylated proteins in frog heart homogenates. Cytosolic (A) and membrane protein fractions (B). CTRL: control; NITRITE: nitrite treated-hearts.

**Table 2**

Different levels of cardiac organization in the vertebrates species examined.

Species	Icefish, frog	Eel
Ventricular myoarchitecture	Trabecular	Trabecular + compact subepicardium
Type of blood supply	Venous, lacunary	Coronary to compact subepicardium, lacunary to innermost myocardium
Interface	EE	EE and VE

EE = endocardial-endothelium; VE = vascular endothelium; Ref. in [49].

Hsp90, a chaperone involved in eNOS activation [42] death receptor-mediated signalling [43], and mitochondrial pro- and anti- apoptotic cascades [44]. Moreover, dysregulated S-nitrosylation of the ryanodine receptor ( $\text{Ca}^{2+}$ -release channel) may contribute to cardiac arrhythmias [45], heat stroke [46], and impaired exercise capacity [47]. In this view, mitochondria deserve particular attention, also in light of the inhibition exerted by nitrite-derived NO on mitochondrial respiration observed by Shiva et al. [23] in both heart homogenate and isolated cardiomyocytes. The present study does not elucidate whether the negative inotropism observed in eel and frog relates to an inhibited mitochondrial respiration. Additional studies are needed in order to clarify whether in the heart of eel, icefish and frog, mitochondria are the final target of nitrite-derived NO. In this context, it should be underlined that, as shown by Shiva et al. [23], in the rat heart nitrite-derived NO reduces mitochondrial respiration through cytochrome c oxidase.

#### 4.1. Conclusions and perspectives

We have shown in non-mammalian hearts with diverse morphologies and blood supply that, under normoxic conditions, nitrite modulates contractility and is also an important intracardiac source of NO. The different nitrite-dependent inotropic responses, which parallel those elicited by authentic NO, uncover at the heart level the already observed species-specific sensitivity to  $\text{NO}_2^-$ , well documented both in freshwater and marine teleosts [25], as well as in several amphibian species [48] after exposure to ambient nitrite. We argue that the comparative approach using different cardiac designs, such as those employed in this work, provides not only remarkable insights on “ancestral” functions of the nitrite-NO system in vertebrates, but also may help to expand its actual significance in human physiology. Indeed, these natural heart models represent powerful research systems that complement the more traditional models of human diseases, like the mouse, holding important clues for understanding fundamental cardiovascular mechanisms. For example, the phylogenetic relationship among Hb-expressing and Hb-less fish families constitutes an ideal matrix of genetic knockouts that can be employed to probe several challenging questions pertinent to the redox biome, including the newly discovered role of Hb and Mb as the predominant nitrite reductases able to generate and regulate NO signalling. By using the heart of the icefish *C. hamatus*, natural knockout for Hb, and an Hb-free perfusion buffer, we demonstrated that Hb is not involved in the reduction of nitrite to NO in the myocardial tissue, thus corroborating the theory [23] that in the heart myoglobin may be the predominant nitrite reductase. In conclusion, comparative studies may pave the way towards an integrated systemic approach concerned with the role of nitrite/NO signalling in vertebrate cardiac physiology, which is also essential for medically-oriented studies and clinical applications.

#### Acknowledgements

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## Phospholamban S-nitrosylation modulates Starling response in fish heart

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# Phospholamban S-nitrosylation modulates Starling response in fish heart

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The Frank–Starling mechanism is a fundamental property of the vertebrate heart, which allows the myocardium to respond to increased filling pressure with a more vigorous contraction of its lengthened fibres. In mammals, myocardial stretch increases cardiac nitric oxide (NO) release from both vascular endothelium and cardiomyocytes. This facilitates myocardial relaxation and ventricular diastolic distensibility, thus influencing the Frank–Starling mechanism.

In the *in vitro* working heart of the eel *Anguilla anguilla*, we previously showed that an endogenous NO release affects the Frank–Starling response making the heart more sensitive to preload. Using the same bioassay, we now demonstrate that this effect is confirmed in the presence of the exogenous NO donor S-nitroso-N-acetyl penicillamine, is independent from endocardial endothelium and guanylate cyclase/cGMP/protein kinase G and cAMP/protein kinase A pathways, involves a PI(3)kinase-mediated activation of endothelial NO synthase and a modulation of the SR-Ca<sup>2+</sup>ATPase (SERCA2a) pumps. Furthermore, we show that NO influences cardiac response to preload through S-nitrosylation of phospholamban and consequent activation of SERCA2a. This suggests that in the fish heart NO modulates the Frank–Starling response through a beat-to-beat regulation of calcium reuptake and thus of myocardial relaxation.

We propose that this mechanism represents an important evolutionary step for the stretch-induced intrinsic regulation of the vertebrate heart, providing, at the same time, a stimulus for mammalian-oriented studies.

**Keywords:** heterometric regulation; myocardial relaxation; endothelial nitric oxide synthase; SERCA2a; stroke volume

## 1. INTRODUCTION

A fundamental property of the vertebrate heart is the Frank–Starling mechanism (heterometric regulation), which allows the myocardium to respond to increased venous return (preload) with a more vigorous contraction of its lengthened fibres, performing more work through augmented stroke volume (SV) and consequent cardiac output (CO). This rapidly occurring stretch-related increase in developed force has been attributed to a length-dependent increase in cross-bridge formation and myofilament calcium responsiveness (Katz 2002).

In mammals, myocardial stretch increases cardiac nitric oxide (NO) release from both vascular endothelium and cardiomyocytes (Pinsky *et al.* 1997; Petroff *et al.* 2001; Balligand *et al.* 2009) facilitating myocardial relaxation, ventricular diastolic distensibility and hence the Starling response (Casadei & Sears 2003). In the past, the effect of NO on preload-induced increases in CO has been attributed to a paracrine effect of endothelial-derived NO on myofilament Ca<sup>2+</sup> sensitivity secondary to troponin I phosphorylation by the cGMP-dependent protein kinase G (PKG) (Prendergast *et al.* 1997). Accordingly, Layland *et al.* (2002) showed that

phosphorylation of troponin I increased in the presence of NO donors, this being associated with increased diastolic cell length and accelerated myocyte relaxation. More recently, an autocrine pathway of NO, related to the specific subcellular localizations and regulation of the different isoforms involved in the production of NO and in its target proteins, has also been described (for references, see Seddon *et al.* 2007). Even though the relative autocrine role of nitric oxide synthase (NOS) isoforms in the cardiomyocyte remains to be fully clarified, there is evidence in mammals that both myocardial endothelial NOS (eNOS) and neuronal NOS (nNOS), through cGMP-independent mechanisms, modulate the preload-induced increase of the contractile force. In details, it has been suggested that the autocrine nNOS, first observed in the sarcoplasmic reticulum (SR) (Xu *et al.* 1999) and subsequently also found bound to sarcolemmal membrane proteins (Williams *et al.* 2006), mainly promotes left ventricular relaxation by regulating the protein kinase A (PKA)-mediated phosphorylation of phospholamban (PLN) and the rate of SR Ca<sup>2+</sup> reuptake by SR Ca<sup>2+</sup> ATPase (SERCA2a) (Zhang *et al.* 2008). On the other hand, myocardial eNOS, mostly expressed into caveolae, a site where several signal transduction pathways have been shown to be modulated by NO (reviewed in Shaul 2002), primarily mediates the inotropic response to sustained stretch through a mechanism which involves S-nitrosylation of thiol residues in the

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Ryanodine receptors  $\text{Ca}^{2+}$  release channels (Massion *et al.* 2005). In addition, Froehlich *et al.* (2008) have recently shown that nitroxyl may stimulate SERCA2a pumps activity by modifying critical thiol residues in its regulatory protein PLN.

As in mammals, also in fishes the end-diastolic volume and the consequent stretch-related increase in developed force is a key regulator of cardiac performance; however, unlike mammals, fishes respond to different hemodynamic loads increasing CO mainly through an increased SV rather than heart rate (HR) (Tota & Gattuso 1996; Olson 1998). This elevated sensitivity of the fish hearts to the Starling response, well documented in both temperate eurytherm and cold-adapted teleosts (gilthead seabream, Icardo *et al.* 2005; eel, Imbrogno *et al.* 2001; icefish, Tota *et al.* 1991), has been in part attributed to a greater myocardial extensibility of the highly trabeculate fish heart, coupled to a maintained increase in myofilament  $\text{Ca}^{2+}$  sensitivity over a large range of sarcomere lengths (Di Maio & Block 2008; Shiels & White 2008).

It is interesting to note we have previously documented that in the eel *A. anguilla* heart, used as a paradigm of a highly trabeculate and endoluminally supplied cardiac design (Tota *et al.* 1983), a basal release of endogenous NO increases the sensitivity of the Frank–Starling response (Imbrogno *et al.* 2001). These studies using avascular working heart preparations, including the amphibian heart (Sys *et al.* 1997), showed that this effect cannot be attributed to a rise in coronary flow or to factors released from vascular tissues. However, neither the NOS isoforms involved nor the mechanism of NO action was directly addressed.

Therefore, the aim of this work was to investigate the mechanism through which NO modulates cardiac performance in the isolated and perfused working heart of the European eel *A. anguilla* under loading conditions. As in previous studies (Imbrogno *et al.* 2001, 2003, 2004, 2006), we took the advantage of using eel hearts in which the heart is mostly perfused endoluminally (lacunary intracardiac supply) so that the nitrenergic influences on myocardial performance can be directly assessed, independently from both the coronary reactivity and the vascular endothelium-produced NO, typical of mammalian paradigms. We demonstrate, to our knowledge, for the first time in a lower vertebrate heart, that the nitrenergic modulation of the Frank–Starling response neither involves the endocardial endothelium (EE)/cGMP/PKG and cAMP/PKA pathways nor the Ryanodine receptors and L-type calcium channels, but acts through an Akt-mediated activation of eNOS-dependent NO production, which through PLN-S-nitrosylation-dependent mechanism can modulate the rate of SR  $\text{Ca}^{2+}$  reuptake and thus myocardial relaxation. Conceivably, this mechanism represents an important evolutionary step for the stretch-induced intrinsic regulation of the vertebrate heart.

## 2. MATERIAL AND METHODS

### (a) Animals

We used specimens of freshwater European eel (*A. anguilla* L.), weighing  $78.5 \pm 2.3$  g (mean  $\pm$  s.e.m.,  $n = 98$ ). Fish were provided by a local hatchery and kept at room temperature ( $18\text{--}20^\circ\text{C}$ ) for 5–7 days. Each eel was

anaesthetized with tricaine methane sulphonate (Sigma Chemical Co., St Louis, MO, USA). In accordance with the accepted standards of animal care, the experiments were organized to minimize stress and number of animal used.

### (b) Isolated and perfused working heart preparations

The hearts, removed and cannulated, were transferred to a perfusion chamber filled with Ringer's solution and connected to a perfusion apparatus as described by Imbrogno *et al.* (2001). Experiments were carried out at room temperature ( $18\text{--}20^\circ\text{C}$ ). For paced experiments, hearts were stimulated with an LE 12006 stimulator (frequency identical to that of control, non-paced hearts; pulse width fixed at 0.1 ms; voltage:  $1.2 \pm 0.1$  V, means  $\pm$  s.e.m.).

### (c) Calculations

HR was calculated from pressure recording curves. CO was collected over 1 min and weighed; values were corrected for fluid density and expressed as volume measurements. The afterload (mean aortic pressure) was calculated as two-thirds diastolic pressure plus one-third maximum pressure. SV ( $\text{ml kg}^{-1}$ ; CO/HR) was used as a measure of ventricular performance; changes in SV were considered to be inotropic effects.

### (d) Experimental protocols

#### (i) Basal conditions

Isolated perfused hearts were allowed to maintain a spontaneous rhythm for up to 15–20 min. In all experiments, the control conditions were a mean output pressure of about 3.00 kPa, with a CO set to  $10 \text{ ml min}^{-1} \text{ kg body mass}$  by appropriately adjusting the filling pressure. These values are within the physiological range (for references, see Imbrogno *et al.* 2001).

#### (ii) Drug application

After the 15–20 min of control period, hearts were perfused for 20 min with Ringer's solution enriched with *S*-nitroso-*N*-acetyl penicillamine (SNAP) or Ryanodine (RYR) or Thapsigargin at increasing concentrations to construct cumulative concentration–response curves.

#### (iii) Frank–Starling response

To assess the interaction between NO and the Frank–Starling response, a Starling curve was generated (baseline condition). After baseline assessment, the input pressure was returned to the control condition and a second Starling curve was generated in the presence of the nNOS inhibitors vinyl-L-*N*-5-(1-imino-3-butenyl)-L-ornithine (L-VNIO) and 7-nitroindazole (7-NI), the NO donor SNAP and its non-nitrosylated parent molecule *N*-acetylpenicillamine (NAP), the phosphatidylinositol-3-kinase (PI3K) antagonist Wortmannin, the cGMP analogue 8-bromoguanosine 3',5'-cyclic monophosphate (8-Br-cGMP), the soluble guanylyl cyclase (sGC) specific inhibitor 1H-(1,2,4)oxadiazole-(4,3-*a*)quinoxalin-1-one (ODQ), the PKG and the PKA antagonists KT<sub>5823</sub> and KT<sub>5720</sub>, respectively, the cGMP-inhibited PDE3 blocker Milrinone, and after inhibition of L-type calcium channels by Diltiazem, or

Ryanodine receptors by RYR, or SERCA2a pumps by Thapsigargin. In addition, the Frank–Starling response was also studied after inducing functional damage of the ventricular EE with the detergent Triton X-100. After baseline curve, 0.1 ml of Triton X-100 at a concentration of 0.05 per cent was injected through a needle inserted into the posterior ventral region of the ventricular wall (for further details, see Imbrogno *et al.* 2001).

The time factor (i.e. the ‘memory’ of the heart) of loading stimulation was excluded according to our previous studies (Imbrogno *et al.* 2001, 2003).

#### (iv) Western blotting

To compare the differences in the protein expression pattern, eel ventricle samples ( $n = 3$  for each condition) were rapidly immersed in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . The ventricles were prepared according to Amelio *et al.* (2006). The proteins were separated on 6 per cent SDS–PAGE gels (for eNOS and phospho-eNOS detection) or 15 per cent SDS–PAGE gels (for phospho-Ser<sup>16</sup>PLN), transferred to membrane, blocked with non-fat-dried milk and incubated overnight at  $4^{\circ}\text{C}$  with polyclonal rabbit anti-eNOS antibody (Sigma) or polyclonal rabbit anti phospho-eNOS antibody (Santa Cruz Biotechnology) or polyclonal rabbit anti phospho-Ser<sup>16</sup>PLN antibody (Santa Cruz Biotechnology) diluted 1:500 in TBS-T containing 5 per cent non-fat dry milk. The anti-rabbit secondary antibody peroxidase linked (Amersham) was diluted 1:5000 in TBS-T containing 5 per cent non-fat dry milk.

#### (v) Biotin switch assay

Ventricles were homogenized on ice in 20 mM Tris pH 7.5, 150 mM NaCl, 1 per cent Igepal CA 630, 0.5 per cent sodium deoxycholate, 1 mM EDTA, 0.1 per cent SDS, 200 mM sodium orthovanadate and Protease Inhibitor Cocktail, using a polytron tissue grinder. The homogenate was centrifuged at  $4^{\circ}\text{C}$  for 40 min at 13 000g. The supernatant containing cytosolic proteins was collected and proteins were quantified with Bradford reagent; the pellet containing membrane proteins was resuspended in homogenization buffer and proteins were quantified with Bradford reagent.

The biotin switch assay was performed as previously described (Jaffrey & Snyder 2001).

To detect biotinylated proteins, samples from the biotin switch assay were separated on 15 per cent SDS–PAGE gels, transferred to membrane, blocked with non fat dried milk and incubated with streptavidin-peroxidase diluted 1:5000 for 1 h. In additional experiments, the membrane for S-nitrosylation detection was stripped and reprobbed using an anti-PLN antibody (Santa Cruz Biotechnology).

#### (vi) Immunodetection and densitometric analysis

The immunodetection was performed using an enhanced chemiluminescence kit (ECL PLUS, Amersham). Autoradiographs were obtained by exposure to X-ray films (Hyperfilm ECL, Amersham). Immunoblots were digitalized and the densitometric analysis of the bands was carried out using NIH IMAGE 1.6 for a Macintosh computer based on 256 grey values (0 = white; 256 = black).

#### (e) Statistics

Percentage changes were evaluated as means  $\pm$  s.e.m. of percentage changes obtained from individual experiments. Because each heart acted as its own control, the statistical significance of differences within group was assessed using the paired Student’s *t*-test ( $p < 0.05$ ). Comparisons between groups were made using two-way analysis of variance (ANOVA). Significant differences were detected using Duncan’s multiple-range test ( $p < 0.05$ ).

The results of absorbance measurements and the grey values obtained from the densitometric analysis were expressed as means  $\pm$  s.e.m. of determinations for each sample. To test the difference between the groups, Student’s *t*-test was performed. Statistical significance was established at  $p < 0.001$ .

#### (f) Drugs and chemicals

8-Br-cGMP, ODQ, KT<sub>5720</sub>, NAP and Triton X-100 were purchased from Sigma Chemical Company. RYR, Wortmannin, Diltiazem, KT<sub>5823</sub> and Thapsigargin were purchased from Calbiochem (Milan). L-VNIO, 7-NI and SNAP were purchased from Vinci-Biochem (Florence).

### 3. RESULTS

#### (a) Effects of S-nitroso-N-acetyl penicillamine and nitric oxide synthase inhibition

We have previously reported that intracardiac NO increases the sensitivity of the *in vitro* eel heart to filling pressure changes. This was demonstrated by the reduction of the preload-induced increases of SV obtained in the presence of the NOS inhibitor L-N<sup>5</sup>(1-iminoethyl) ornithine (L-NIO) (Imbrogno *et al.* 2001). To establish whether exogenous NO also affects the preload response, this was evaluated before and after treatment with the NO donor SNAP ( $10^{-9}$  M). Pre-treatment with SNAP induced a significant increase of Starling response (figure 1a). Of note, the non-nitrosylated parent molecule of SNAP, NAP ( $10^{-9}$  M) did not modify Starling response (at the max preload of 0.6 kPa, the percentage increment of SV was  $214 \pm 32\%$  in NAP-treated hearts versus  $222 \pm 28\%$  for control). Under basal conditions, SNAP decreased SV, the effect being significant from  $10^{-11}$  M (figure 1b).

To discriminate the NOS isoform involved in the NO-dependent modulation of Frank–Starling response, heart preparations were exposed to two specific nNOS antagonists, L-VNIO ( $10^{-5}$  M) and 7-NI ( $10^{-5}$  M). nNOS inhibition did not influenced the response to preload changes, ruling out the involvement of nNOS and supporting a role for eNOS. At the max preload of 0.6 kPa, the percentage increments of SV were:  $154 \pm 22\%$  in L-VNIO-treated hearts versus  $167 \pm 19\%$  for control;  $148 \pm 22\%$  in 7-NI-treated hearts versus  $172 \pm 28\%$  for control.

#### (b) Role of endocardial endothelium-dependent nitric oxide production

The heart of *A. anguilla* possesses a highly trabeculated ventricle with an extensive EE surface that, being an important source of NO, modulates basal and chemically stimulated cardiac performance (Imbrogno *et al.* 2001,

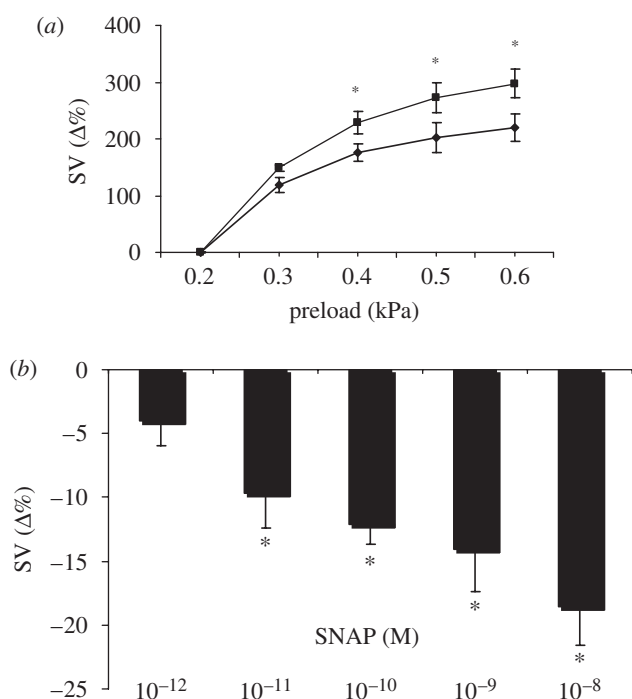


Figure 1. (a) Effect of preload elevation on SV under control conditions (filled diamonds) and after treatment with the NO donor SNAP (10<sup>-9</sup> M, filled squares). Percentage changes were evaluated as mean  $\pm$  s.e.m. ( $n = 5$ ). Comparison between groups was made using two-way ANOVA analysis,  $*p < 0.05$ . (b) Dose–response curve for SNAP (10<sup>-12</sup>–10<sup>-8</sup> M). Percentage changes were evaluated as means  $\pm$  s.e.m. ( $n = 4$ ). Significance of differences from control values ( $t$ -test);  $*p < 0.05$ .

2003). To assess the role of EE-derived NO on the eel heart response to preload, double Starling curves were generated in the presence of Triton X-100. The EE impairment caused by Triton X-100 (0.05%) did not modify the response to preload increases, thus excluding an EE source of NO in the short-term nitrenergic modulation of Starling response (at the max preload of 0.6 kPa, the percentage increment of SV was  $206 \pm 38\%$  in Triton-treated hearts versus  $207 \pm 21\%$  for control).

### (c) Preload-induced endothelial nitric oxide synthase activation

It has been reported in mammals that stretching of cardiac muscle induces a PI3K-dependent phosphorylation of the eNOS (Petroff *et al.* 2001). This mechanism, which involves membrane caveolae (regarding their occurrence in fish heart, see Di Maio & Block 2008) and cytoskeleton (Casadei & Sears 2003), causes a sustained increase in enzyme activity, probably by reducing the dissociation of calmodulin from activated eNOS (McCabe *et al.* 2000), thus enhancing NO production. To verify our hypothesis, i.e. the PI3K involvement in the preload-induced NO production, we studied the Frank–Starling response before and after treatment with the PI3K antagonist Wortmannin (10<sup>-9</sup> M). The results obtained showed a significant decrease of Starling response, suggesting that also in the eel, mechanical stimuli could activate, via a PI3K-dependent pathway,

the release of NO, which in turn modulates the cardiac heterometric mechanism (figure 2a).

### (d) Phospho-endothelial nitric oxide synthase expression

Western blotting analysis showed the presence of phospho-eNOS in the hearts of *A. anguilla* either under basal conditions (physically unstimulated hearts) or after Starling response or after Starling response performed in the presence of Wortmannin (10<sup>-9</sup> M). In fact, an immunoreactive band of approximately 140 kDa, corresponding to the known p-eNOS molecular weight, was detected. The p-eNOS expression has been normalized by Western blotting analysis performed on the same extracts incubated with polyclonal anti-eNOS antibody (figure 2b). Densitometric quantification of the blots revealed an increase of 22 per cent ( $p < 0.001$ ) of phospho-eNOS expression after the Starling response with respect to basal conditions. This value significantly decreased by 17 per cent ( $p < 0.001$ ) when Starling curves were performed after Wortmannin treatment, showing a non-significant decrement of 1.5 per cent with respect to basal conditions (figure 2c).

### (e) Nitric oxide intracellular signalling

#### (i) Role of cyclic guanosine monophosphate-mediated pathways

In the heart, NO is known to activate sGC to produce cGMP, which in turn may signal through the cGMP-dependent PKG or by modulating PKA via the cGMP-modulated phosphodiesterases of cAMP (PDE). We have previously shown that also in the working eel heart, intracardiac NO exerts an important modulation of mechanical performance via sGC signalling (see Imbrogno *et al.* 2001, 2003, 2004, 2006). Of note, treatments with either a specific inhibitor of sGC ODQ (10<sup>-5</sup> M), or with the cGMP-activated PKG inhibitor KT<sub>5823</sub> (10<sup>-6</sup> M), or with cGMP analogue 8-Br-cGMP (10<sup>-6</sup> M), or with KT<sub>5720</sub> (10<sup>-6</sup> M) had no effects on the Frank–Starling response. At the max preload of 0.6 kPa, the percentage increments of SV were:  $174 \pm 21\%$  in ODQ-treated hearts versus  $198 \pm 23\%$  for control;  $176 \pm 20\%$  in 8-Br-cGMP-treated hearts versus  $164 \pm 11\%$  for control;  $180 \pm 16\%$  in KT<sub>5823</sub>-treated hearts versus  $200 \pm 8\%$  for control;  $204 \pm 45\%$  in KT<sub>5720</sub>-treated hearts versus  $211 \pm 20\%$  for control (see the corresponding figure in the electronic supplementary material). Similarly, block of the cGMP-inhibited PDE3 with Milrinone (10<sup>-6</sup> M) (data not shown) had no influence on the eel heart response to preload increases. Taken together, these findings exclude the involvement of cGMP signalling downstream of myocardial NO production in the nitrenergic modulation of the Frank–Starling response.

#### (ii) Role of calcium

Recent literature in mammals has designed NO as a key modulator of Ca<sup>2+</sup> cycling in the stretched myocytes. Under stretch conditions, NO through S-nitrosylation of reactive thiols has been shown to stimulate L-type Ca<sup>2+</sup> channel function (Sun *et al.* 2006) and to enhance the Ryanodine receptor open probability (Petroff *et al.* 2001). Moreover, by activating SERCA2a pumps, it can

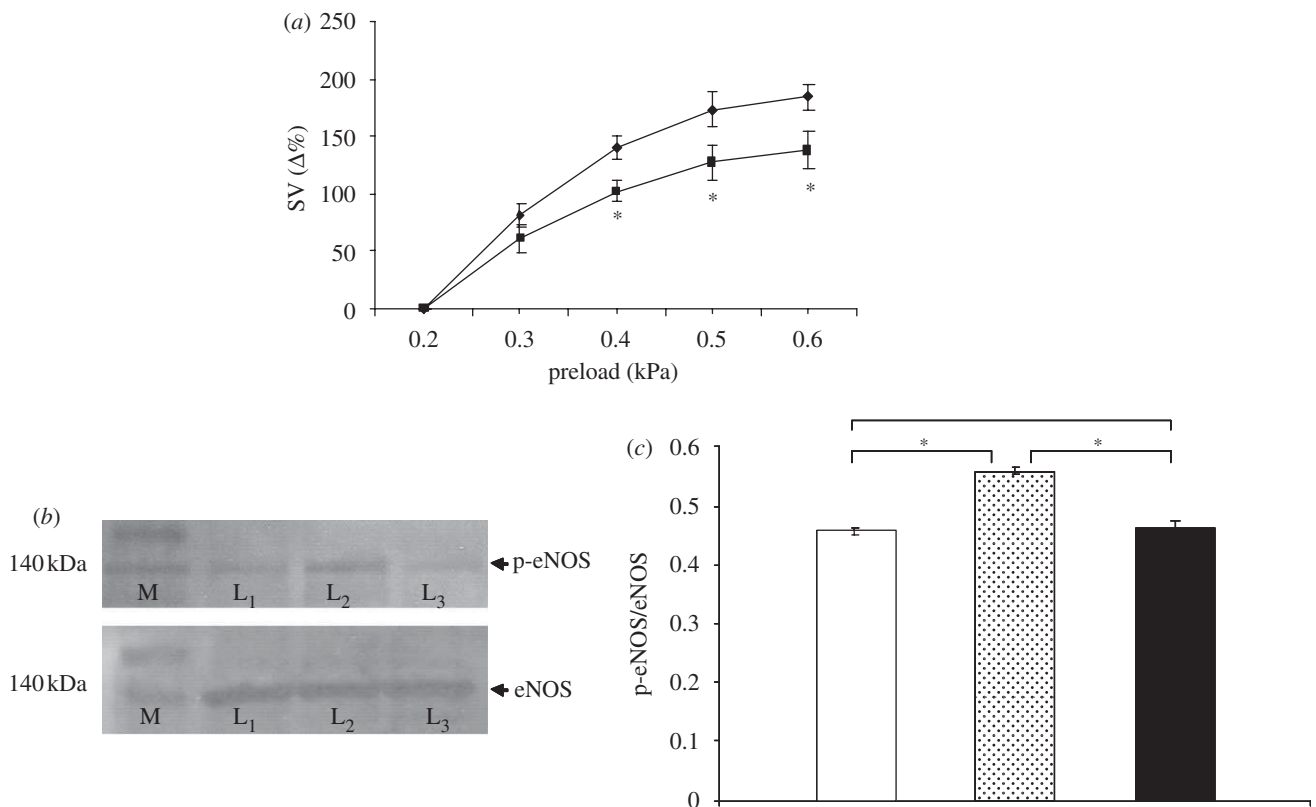


Figure 2. (a) Effect of preload on SV under control conditions (filled diamond) and after treatment with Wortmannin ( $10^{-9}$  M, filled square). Percentage changes were evaluated as mean  $\pm$  s.e.m. ( $n = 5$ ). Comparison between groups was made using two-way ANOVA analysis,  $*p < 0.05$ . (b) Western blotting of p-eNOS and total eNOS in heart extracts (M, marker; L<sub>1</sub>, basal conditions; L<sub>2</sub>, Starling response; L<sub>3</sub>, Starling response in the presence of Wortmannin). (c) Densitometric quantification of p-eNOS over total eNOS ratio (unfilled bar, basal conditions; grey bar, Starling response; black bar, Starling + Wortmannin). Data are means  $\pm$  s.e.m. of five determinations for each animal ( $n = 3$ ). Statistical differences were evaluated by Student's *t*-test;  $*p < 0.001$ .

increase calcium reuptake into the SR (Massion *et al.* 2005).

In the eel heart, the role of  $\text{Ca}^{2+}$  in response to preload increases was pharmacologically tested before and after treatment with inhibitors of either L-type calcium channels (Diltiazem,  $10^{-7}$  M) or Ryanodine receptors (R<sub>YR</sub>,  $10^{-7}$  M) or SERCA2a pumps (Thapsigargin,  $10^{-7}$  M). While R<sub>YR</sub> and Diltiazem pre-treatments did not influence the preload-induced increases in SV, Thapsigargin significantly reduced them, suggesting that the nitroergic modulation of the Frank–Starling response in the eel heart occurs via an NO modulation of the rate of SR  $\text{Ca}^{2+}$  reuptake (figure 3*a–c*). Under basal conditions, R<sub>YR</sub> significantly decreased SV at the concentration of  $10^{-7}$  M (data not shown); on the contrary, Thapsigargin *per se* did not modify basal mechanical performance (figure 3*d*). Concentration–response curves for Diltiazem significantly decreased SV from the concentration of  $25 \times 10^{-9}$  M (Imbrogno *et al.* 2004).

#### (f) Analysis of S-nitrosylated proteins

To assess whether the Starling response induces protein S-nitrosylation in the perfused hearts, using the biotin switch method, we analysed the pattern of proteins containing S-nitrosylated cysteines in homogenates of control hearts and Starling-treated hearts. The analysis of S-nitrosylated proteins in the eel cardiac tissues clearly revealed that stretch increases the degree of S-nitrosylation

of a broad range of proteins. This range includes the protein band that migrates at the same location where PLN was determined by Western blot, as a monomer (6 kDa), dimer (12 kDa) and pentamer (30 kDa) (figure 4*a*). Densitometric quantification of the blots revealed statistically significant differences between control hearts and preload-treated hearts (figure 4*b*). The increment in the S-nitrosylation signal at this precise location was corroborated using an anti-PLN antibody (figure 4*c*).

#### (g) Phospho-Ser<sup>16</sup>-phospholamban expression

Western blotting analysis showed the phosphorylation of PLN in the Starling-treated hearts. A band of approximately 6 kDa, corresponding to the PLN monomer molecular weight, was detected (figure 4*d*). The densitometric analysis of the intensity signal is shown in figure 4*e*.

## 4. DISCUSSION

Using the *in vitro* working heart of the eel *A. anguilla*, in which the influences of coronary reactivity and vascular-generated autacoids (e.g. NO) are excluded, we demonstrate that the endogenous NO-induced modulation of the Frank–Starling response involves an Akt-mediated activation of eNOS, is independent from the cGMP/PKG and cAMP/PKA pathways, as well as from Ryanodine receptors and L-type calcium channels,



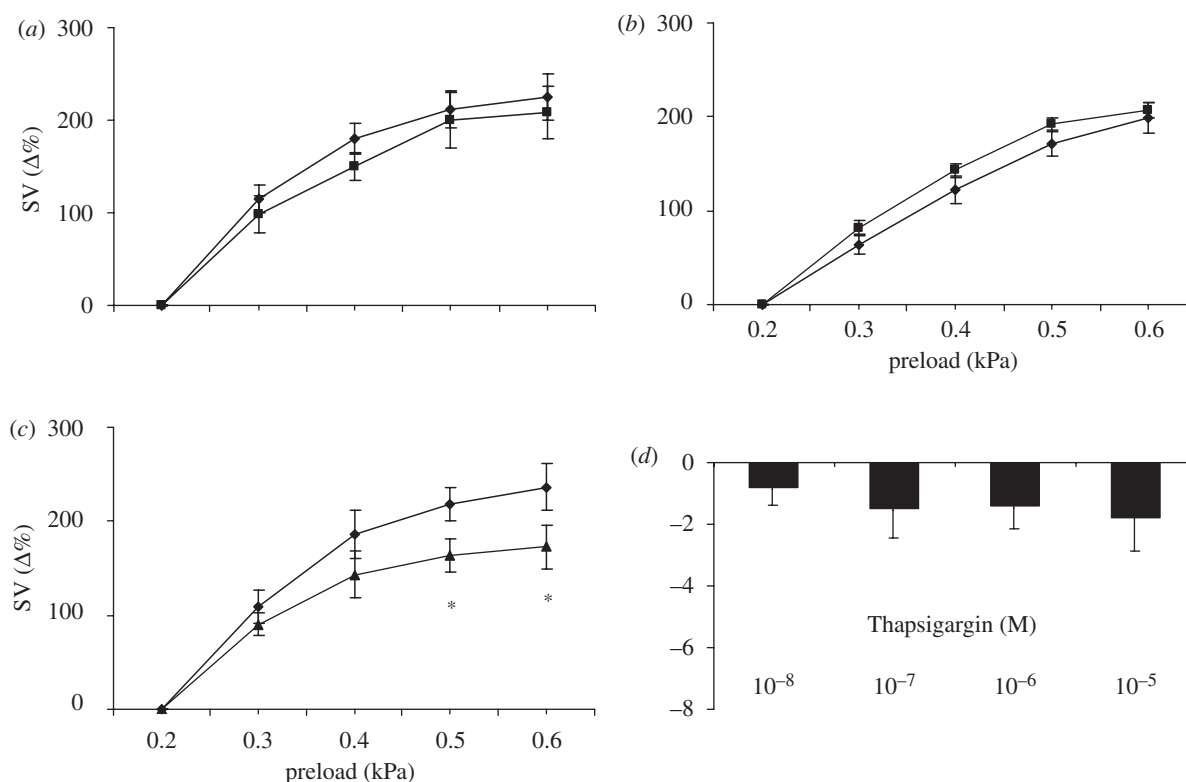


Figure 3. Effect of preload on SV under control conditions (filled diamonds) and after treatment with (a) Diltiazem ( $10^{-7}$  M) (filled squares), (b) RYR ( $10^{-7}$  M) (filled squares) and (c) Thapsigargin ( $10^{-7}$  M) (filled triangles). Percentage changes were evaluated as mean  $\pm$  s.e.m. ( $n = 5$  for each group). Comparison between groups was made using two-way ANOVA analysis,  $*p < 0.05$ . Dose–response curve for (d) Thapsigargin ( $10^{-8}$ – $10^{-5}$  M). Percentage changes were evaluated as mean  $\pm$  s.e.m. ( $n = 4$ ).

but functions through an S-nitrosylation of PLN with consequent activation of SERCA2a pumps.

#### (a) Role of exogenous nitric oxide

Under basal conditions (physically unstimulated hearts), treatment with SNAP (from  $10^{-12}$  to  $10^{-8}$  M) elicited a concentration-dependent decrease of SV, significant from the concentration of  $10^{-11}$  M. This result confirms previous findings (Imbrogno *et al.* 2001), showing that another NO donor, 3-morpholinosydnonimine (SIN-1), at the same concentrations induced effects of similar magnitude. The confirmation in the eel heart that NO tonically modulates mechanical performance in absence of loading and agonists stimulations is an important point in view of the apparently contrasting findings on the NO-induced contractile myocardial effects, probably owing to species-related differences and also to problems related to the kind of experimental design employed (Shah 1996; Tota *et al.* 2005). The lower vertebrate avascular heart preparations, working ‘physiologically’ (reproducing the haemodynamic responses of the *in vivo* heart), free of extrinsic nervous and humoral influences, appear well suited to analyse directly the cardiac auto-crine/paracrine NO role (Imbrogno *et al.* 2001, 2003; Acierio *et al.* 2008).

The significant differences between the Starling curves obtained with and without the exogenous NO donor SNAP ( $10^{-9}$  M), evaluated by two-way ANOVA analysis, but not with the non-nitrosylated analogue of SNAP, NAP ( $10^{-9}$  M), highlight the relevance of the nitrenergic modulation of the cardiac heterometric mechanism in

the eel. Noteworthy, however, some controversy about the interpretation of the NO-elicited effects has been recently related to the use of NO donors as experimental tools, since these agents may not necessarily reproduce the physiological effects of the constitutively expressed NOSs, which generate NO in a temporally and spatially restricted manner and also depend upon the specific stimulus for NO release (Zhang *et al.* 2008 and references therein). Therefore, to study the NO-activated downstream transduction mechanism in response to stretch, we focused our attention on the endogenously produced NO, deliberately avoiding the use of NO donors.

#### (b) Stretch-dependent activation of endothelial nitric oxide synthase

In mammals, the stretch-induced NO modulation of cardiac performance has been ascribed to NO generation by both the constitutive NOS isoforms (eNOS and nNOS) (Massion *et al.* 2005). In the eel, we observed that while pre-treatment with an unspecific NOS inhibitor, L-NIO, significantly reduced the preload-induced increase in SV (see Imbrogno *et al.* 2001), this was not influenced by the two nNOS antagonists L-VNIO and 7-NI, ruling out the nNOS involvement while supporting a role for eNOS. In line with these results, Tota *et al.* (2005) reported the presence of eNOS, but not of nNOS, in the ventricular myocardium of the eel. Although these results do not allow us to exclude the presence of nNOS in the eel myocytes, they, however, suggest an exclusive involvement of eNOS in the Frank–Starling response. Interestingly, while myocardial nNOS is considered to exert a primary

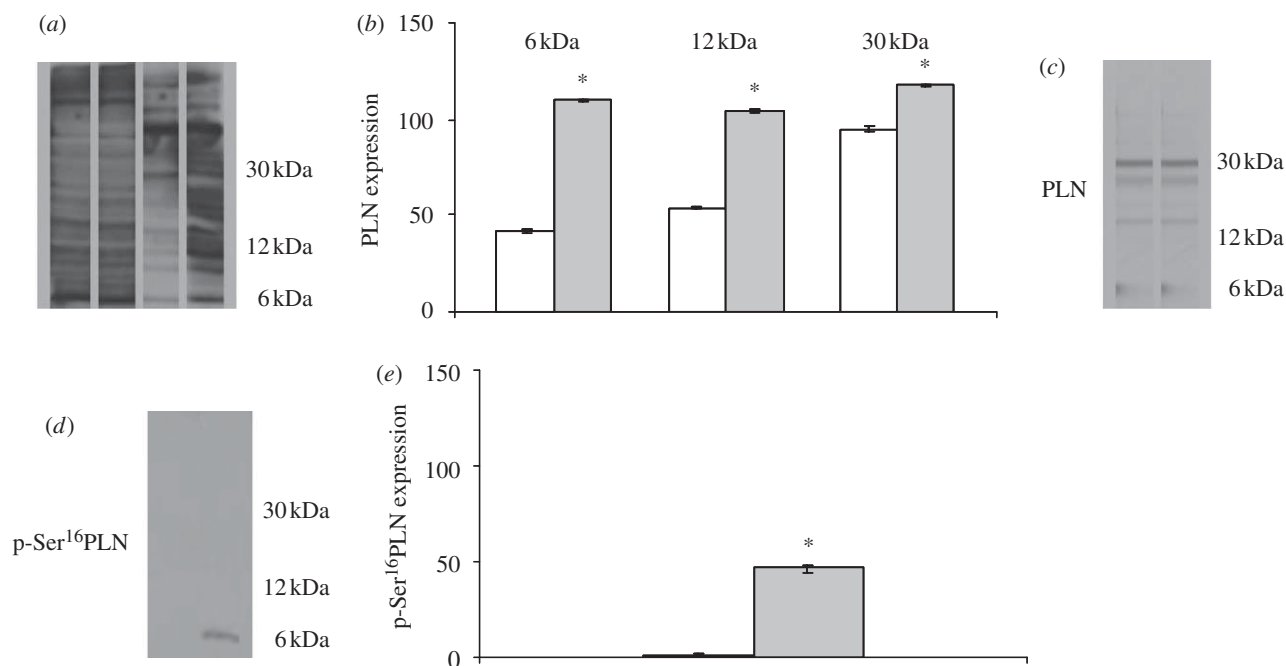


Figure 4. (a) Western blot of S-nitrosylated proteins in heart homogenates: cytosolic (lanes 1 and 2) and membrane (lanes 3 and 4) protein fractions; control heart (lanes 1 and 3) and Starling-treated heart (lanes 2 and 4). (b) Amount of S-nitrosylation at the migration position corresponding to the PLN as a monomer (6 kDa), dimer (12 kDa) and pentamer (30 kDa). (c) Membrane stripped and incubated with anti PLN antibody. Western blot of (d) phospholamban phosphorylated at Ser16 and (e) densitometric analysis. Data are means  $\pm$  s.e.m. of five determinations for each animal ( $n = 3$ ). Statistical differences were evaluated by Student's *t*-test;  $*p < 0.001$ . (b) and (e) Unfilled bar, control; grey bar, Starling.

role in the regulation of basal and adrenergically stimulated contractions, eNOS seems preferentially activated by mechanical stimuli, acting as a major myocardial regulator in the stretch-induced response (Sears *et al.* 2004 and references therein).

The exact mechanism responsible for the stretch-induced augmentation of NO production in mammals has not yet been completely elucidated, however there is evidence suggesting an implication of a tyrosine kinase inhibitor-sensitive pathway, involving membrane caveolae and cytoskeleton (Fleming & Busse 1999). In the teleost heart, the presence of caveolae, junctional SR profiles and the network of free SR has been described in detail by Di Maio & Block (2008). It is known that the serine-threonine kinase Akt phosphorylates eNOS *in vitro* as well as *in vivo* (Dimmeler *et al.* 1999), thereby causing a sustained increase in the enzyme activity (McCabe *et al.* 2000). Also nNOS possesses an Akt phosphorylation motif, but the phosphorylation effects on enzyme activity are inconsistent or mostly inhibitory (Dinerman *et al.* 1994). On the basis of this mammalian framework, we found that in the eel heart, the nitrenergic modulation of the Starling response involves a PI3K-Akt-mediated phosphorylation of eNOS. This was demonstrated by the significant reduction of Starling response observed after inhibition of PI3K by Wortmannin pre-treatment and further confirmed by Western blotting analysis on heart homogenates incubated with anti phospho-eNOS antibody, showing a significant increase in the phosphorylated isoform in Starling-treated hearts with respect to the basal conditions. In addition, the loss of phospho-eNOS expression increment in the Starling-treated hearts in presence of Wortmannin strongly supports the hypothesis of a stretch-induced PI3K-Akt-eNOS pathway.

#### (c) Intracellular signalling and nitric oxide downstream effectors

Several *in vitro* (Paolocci *et al.* 2000) and intact animal (Preckel *et al.* 1997) studies suggested that both cGMP-dependent and -independent mechanisms contribute to the NO-mediated influence on myocardial function. NO activates sGC by binding to its haeme moiety, leading to cGMP production, consequent PKG activation and a cascade of biological signalling events (Layland *et al.* 2002). Moreover, NO can react with thiol residues of numerous compounds or proteins. cGMP/PKG activation is considered a major transduction pathway for NO cardiac regulation, whereby, among other effects, it induces myofilament desensitization to  $Ca^{2+}$ , thought to be responsible for accelerated myocardial relaxation through troponin I phosphorylation at Ser23/24 (Layland *et al.* 2002). In the working eel heart, the NO-induced cGMP-PKG pathway remarkably modulates mechanical performance (see, for references, Tota *et al.* 2005). For example, it tonically decreases SV either under basal condition (Imbrogno *et al.* 2001) or following endoluminal chemical stimulations of AT<sub>1</sub> angiotensin II receptors (Imbrogno *et al.* 2003), or  $\beta_3$  adrenergic receptors (Imbrogno *et al.* 2006), or exposure to the anti-adrenergic cardio-inhibitory peptide vasostatin I (Imbrogno *et al.* 2004). Importantly, however, the present data strongly suggest that the cGMP-PKG mediated mechanisms are not involved in the eel heart under loading conditions, since the Frank-Starling response is not influenced by pre-treatment with either the sGC inhibitor ODQ or the cGMP analogue 8-Br-cGMP or the PKG antagonist KT<sub>5823</sub>. This, together with the data that exclude the involvement of the EE in the stretch-induced NO production, implies the activation of different cardiac

NO-signal processes, such as those related to NOS isoforms compartmentation and differences in their mode of stimulation, as well as the diffusion distance of NO within intra-myocyte molecular targets and final effectors, all of which providing now the rationale for resolving previous contradictions existing in NO cardiac biology (Seddon *et al.* 2007).

Recent evidence in mammals has designated NO as a key modulator of  $\text{Ca}^{2+}$  cycling in the stretched myocytes. In rat LV myocytes, stretch-induced increase in intracellular  $\text{Ca}^{2+}$  transient appears correlated to an increase in Ryanodine receptors open probability and SR  $\text{Ca}^{2+}$  release, an effect attributed to a direct S-nitrosylation of reactive thiols associated with a stretch-induced NO production (see Casadei & Sears 2003 for references). NO-induced regulation of calcium entry on a beat-to-beat basis is also suggested by S-nitrosylation of thiols on L-type  $\text{Ca}^{2+}$  channels associated with modulation of their calcium current and of the amplitude of contractile shortening (Sears *et al.* 2003). That myocardial NO production by nNOS and/or eNOS may tonically stimulate SERCA2a activity is also supported by an NO-dependent myocardial relaxation associated with SERCA2a-induced increase in calcium reuptake into the SR (Massion *et al.* 2005), as well as by a decrease in both SR  $\text{Ca}^{2+}$ -ATPase activity and  $\text{Ca}^{2+}$  uptake in SR microvesicles from nNOS and/or eNOS knockout mice (Zhou *et al.* 2002).

Interestingly, the idea that NO may influence SERCA2a activity is also now supported in the eel heart by our results, which therefore suggest that this NO-signalling mechanism has been maintained over evolutionary time, being properly integrated into an established developmental and physiological cardiac system. In fact, we observed that the preload-induced increases in SV were not significantly affected by pre-treatment with Diltiazem or RYR, excluding a role for both L-type and RYR calcium channels, respectively, while they were significantly reduced by Thapsigargin-dependent inhibition of SERCA2a pumps. Although calcium transients were not directly measured in this study, we hypothesize that the effects of NO on the stretch-dependent increase in SV is primarily attributable to a quickening of calcium removal and thus of muscle relaxation.

The cardiac Ca-ATPase, SERCA2a, is regulated by PLN, a small membrane protein which, in its dephosphorylated state, inhibits SR  $\text{Ca}^{2+}$  sequestration by SERCA2a (Kimura *et al.* 1997). As supported by mutagenesis studies, the PLN monomer is the active isoform, dissociates from the pentamer, which acts as a reservoir of monomers, binds to SERCA2a and inhibits the pump by direct protein interaction (Reddy *et al.* 1999). PLN phosphorylation at Ser16 by PKA relieves its inhibitory action on SERCA2a, causing an increased rate of myocardial relaxation (Schmidt *et al.* 2001). However, recent evidence suggests an alternative, phosphorylation-independent mechanism of SERCA2a-regulated  $\text{Ca}^{2+}$  reuptake. In fact, Froehlich *et al.* (2008) showed that activation of SERCA2a could be achieved by modifying critical thiol residues in the PLN, which through protein conformation change relieves the inhibition of the pump. In particular, the covalent adduction of a nitroso group to a cysteine thiol side chain has recently emerged as a major mechanism by which NO mediates a large number of intracellular processes (Hess *et al.* 2005) and an increase in proteins S-nitrosylation by NO was recently observed, also in eel cardiac tissues (Cerra *et al.* 2009).

In the fish heart, both the presence of PLN in myocyte SR and the role of PLN phosphorylation in SERCA2a activation have been reported (Will *et al.* 1985; Castilho *et al.* 2007), but the role of PLN S-nitrosylation in the pump activation is heretofore completely unknown. As in mammals, also in fish, the amino acid sequence of PLN (see GenBank accession numbers for zebrafish: XM\_701636.1 and puffer fish: CAG06667, DeWitt *et al.* 2006) includes cysteine residues, which are a potential target for S-nitrosylation. Using the biotin switch method (Jaffrey & Snyder 2001), we demonstrated, to our knowledge, for the first time in a non-mammalian heart, that preload increases can induce a S-nitrosylation of PLN. Moreover, Western blotting analysis showed a Starling-induced phosphorylation of PLN monomeric form (figure 4d). These results suggest that in fish heart, in conjunction with the classical PLN phosphorylation-dependent pathway, the SERCA2a pump catalytic efficiency can be directly modulated by NO through a mechanism which involves PLN S-nitrosylation. The possibility that, in addition to a nitrosylation of PLN, NO may directly inactivate specific phosphatases (Zhang *et al.* 2008) could also account for the Starling-induced phosphorylation of PLN. This issue challenges further study.

In conclusion, the present study demonstrates the existence of an important nitric modulation of the Frank–Starling response in the fish heart, which occurs via a cGMP-independent pathway, involves an Akt-mediated activation of eNOS-dependent NO production that, in turn, modulates the rate of SR  $\text{Ca}^{2+}$  reuptake through PLN S-nitrosylation. This supports the hypothesis that in the fish heart the ‘beat-to-beat’ regulation, i.e. the principal mechanism of intrinsic regulation of CO, is directly modulated by myocardial autocrine NO through a non-classical signalling that by-passes the activation of transduction cascades and the involvement of second messengers. Therefore, the challenging question arises whether, and to what extent, this mechanism represents an old evolutionary fish-specific or, instead, a well conserved (for its importance) universal trait of the vertebrate heart. Since, despite the rapidly growing number of data published on NO biology during the last 20 years, about 99 per cent of the studies are referred to (few) mammalian species (Moroz & Kohn 2007), here we propose that the investigation of evolutionary models, commonly recognized as ‘non-model species’, can provide highly useful natural tools to enhance our multilevel phylogenetic and molecular understanding regarding the involvement of myocardial S-nitrosylation processes in the NO-modulated regulation of the vertebrate heart.

In accordance with the accepted standards of animal care, the experiments were organized to minimize stress and number of animals used.

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